DESCRIPTION

AN OCIF-BINDING MOLECULE (OBM), NUCLEIC ACID ENCODING, AND PROCESS FOR PRODUCING THE SAME

TECHNICAL FIELD

The present invention relates to a novel protein (OCIF binding molecule; hereinafter it may be referred to as "OBM"), which binds osteoclastogenesis inhibitory factor, and a production method thereof. In addition, the present invention also relates to DNA which encodes the protein, a protein having an amino acid sequence encoded by the DNA, a method for genetically producing the protein, and a pharmaceutical composition comprising the protein.

The present invention also relates to a methods of screening for: a substance which controls expression of the protein, a substance which inhibits or modifies the biological activity of the protein, or a receptor which binds the protein and transmits the activity thereof, methods of using the protein or the DNA; the substances obtained by these methods; and pharmaceutical compositions comprising the obtained substances. In addition, the present invention also relates to an antibody to the protein, a method for the production thereof, a method for measuring the protein with the antibody, and an agent comprising the antibody.

BACKGROUND ART

Bone metabolism depends on the overall activity of osteoblasts responsible for bone formation, and osteoclasts, responsible for bone resorption. It is assumed that bone metabolism abnormality is caused due to loss of balance between bone formation and bone resorption. As diseases involving bone metabolism abnormality, osteoporosis, hypercalcemia, bone Paget's disease, renal osteodystrophy, rheumatoid arthritis and osteoarthritis are known. A representative of these bone metabolism abnormality diseases is osteoporosis. This disease occurs when bone resorption by osteoclasts exceeds bone formation by osteoblasts and is characterized by equal decrease in bone calcareous substances and bone matrix. The mechanism for crisis of this disease is not yet fully clarified, while it is a disease with pain in bone and bone fracture due to the increased fragility of bone. Along with an increase in the population of aged people, this disease causes aged people to fracture bone, resulting in confinement in bed. This disease is already a social problem, so that medicaments for treating the disease are urgently needed to be developed. It is expected that osteopenia due to bone metabolism abnormality can be treated

by stimulating bone formation, inhibiting bone resorption, or improving the balance between them. That is, bone formation is expected be stimulated by promoting the growth, differentiation and functions of osteoblasts, which are responsible for bone formation, suppressing the differentiation of osteoclast precursor cells to osteoclasts and maturation thereof, or suppressing osteoclast function such as bone-resorbing activity. At present, hormones, substances of low molecular weight or physiologically active proteins having such activity are being studied and developed.

As agents for treating bone-relating diseases and shortening treatment periods thereof, a calcitonin-containing formulation, the active-form of vitamin D_3–containing formulation, hormone (estradiol, ipriflavone, vitamin K_2) –containing formulation and bisphosphonate-based compound are already clinically available. Furthermore, to develop medicaments with less side effects and excellent effectiveness, clinical trials of the active-form of vitamin D_3 derivatives, estradiol derivatives, and bisphosphonate-based compounds of the second or third generation have been held.

However, since such methods for treatment using these drugs are not necessarily sufficient in effectiveness and results of treatment, novel medicaments that are safer and have higher effectiveness have been expected to be developed. Moreover, among medicaments used in treatment of bone metabolism diseases, there are those which can be used only for treating a restricted kind of disease due to the side effects thereof. In addition, at present, to treat bone metabolism diseases such as osteoporosis, treatment with combined use of more than one medicament is currently usual. From such a point of view, a medicament having different action mechanisms from those of the conventional ones with higher effectiveness and less side effects have been expected to be developed.

As described above, cells responsible for bone metabolism are osteoblasts and osteoclasts. It is known that these cells closely interact with each other, and this phenomenon is regarded as coupling. That is, it has been reported that the differentiation and maturation of osteoclasts are stimulated or suppressed by cytokines, interleukins 1 (IL-1), 3 (IL-3), 6 (IL-6) and 11 (IL-11), granulocyte-macrophage colony-stimulating factors (GM-CSF), macrophage colony-stimulating factors (GM-CSF), interferon gammas (IFN-γ), tumor necrosis factors α (TNF-α), transforming growth factors β (TGF-β) and the like, which are secreted from osteoblast-like stroma cells (Raisz: Disorders of Bone and Mineral Metabolism, 287 to 311, 1992; Suda *et al.*: Principles of Bone Biology, 87 to 102, 1996; Suda *et al.*: Endocrine Reviews, 4, 266 to 270, 1955, Lacey *et al.*: Endocrinology, 186, 2369 to 2376, 1995). It is known that osteoblast-like stromal cells play an important role in differentiation and maturation of osteoclasts and expression of mature osteoclast function, such as bone resorption, through intercellular binding to immature precursor cells of

osteoclasts or (mature) osteoclasts. As a factor involved in osteoclastogenesis by the intercellular binding, a molecule known as osteoclast differentiation factor (ODF) (Suda *et al.*: Endocrine Rev. 13, 66 to 80, 1992; Suda *et al.*: Bone 17, 87S to 91S, 1995) which is expressed on the membrane of the osteoblast-like stromal cell is predicted. According to this assumption, a receptor for ODF exists in the osteoclast precursor cell. However, ODF and this receptor are not yet either purified or identified, and there are no reports on their characteristics, action mechanisms or nd structures. As just described, the mechanism for differentiation and maturation of osteoclasts has not been fully understood yet, and it is expected that full understanding of that mechanism will significantly contribute not only to the field of experimental medicines but also to developments of novel agents for treating bone metabolism abnormality, based on the novel action mechanism.

Under the circumstances, the present inventors have made intensive studies and found osteoclastogenesis inhibitory factors (OCIF) in the culture solution of human fetal lung fibroblasts IMR-90 (ATCC CCL186) (WO 96/26217).

Then, the present inventors succeeded in DNA cloning of OCIF, production of a recombinant OCIF using an animal cell, and confirmation of *in vivo* medicinal virtues (bone metabolism improving effect) of the recombinant OCIF. OCIF is expected as a medicament that has higher effectiveness and causes less side effects than the conventional one and can prevent and treat diseases associated with bone metabolism abnormality.

DISCLOSURE OF THE INVENTION

The present inventors have intensively searched for the existence of a protein that binds to osteoclastogenesis inhibitory factor OCIF by using OCIF. As a result, the inventors have found that OCIF binding protein is specifically expressed on an osteoblast-like stromal cell cultured in the presence of bone resorption factors such as the active-form of vitamin D_3 and parathyroid hormone (PTH). Furthermore, as a result of studying the characteristics and physiological functions of OCIF binding protein, the protein was found to have biological activity as a so-called osteoclast differentiation and maturation factor, associated with differentiation of immature osteoclast precursor cells to osteoclasts and maturation thereof. The present invention has been completed based on this finding. Moreover, as a result of further studying the protein of the present invention, the present inventors have found that the novel membrane protein is an important protein which leads the differentiation and maturation of immature osteoclast precursor cells to osteoclasts by osteoblast-like stromal cells in a co-culture system of the osteoblast-like stromal cells and spleen cells. The successful identification, isolation and purification of the protein as a factor which supports and promotes the differentiation and maturation of osteoclasts in the present

invention enables a screening of a novel agent for treating bone metabolism abnormality, based on a mechanism for bone metabolism in a living subject, using the protein of the present invention.

Therefore, an object of the present invention is to provide a novel protein (OCIF binding molecule; OBM), which binds osteoclastogenesis inhibitory factor (OCIF), and a method for the production thereof. Another object of the present invention is to provide DNA which encodes the protein, a protein having an amino acid sequence encoded by the DNA, a method for genetically producing the protein, and a pharmaceutical composition comprising the protein. Furthermore, another object of the present invention is to provide an agent for preventing and/or treating bone metabolism abnormality comprising the protein. Moreover, another object of the present invention is to provide: a method of screening for: a substance which controls expression of the protein, a substance which inhibits or modifies the biological activity of the protein, or a receptor which binds the protein and transmits the activity of the protein; a method of using the protein and DNA thereof; a substance obtained by that method; and pharmaceutical compositions comprising the obtained substance. Furthermore, another object of the present invention is to provide an antibody to the protein, a method for production thereof, a method for measuring the protein using the antibody, and a medicament (agent; pharmaceutical composition) comprising the antibody.

The protein of the present invention shows the following physicochemical properties and biological activity. That is, (a) the protein specifically binds osteoclastogenesis inhibitory factor (OCIF) and has high affinity (a dissociation constant, a Kd value, on a cell surface, is not larger than 10^{-9} M); (b) the protein shows a molecular weight of about 30,000 to 40,000 as measured by SDS-polyacrylamide electrophoresis under non-reducing conditions, and shows an apparent molecular weight of about 90,000 to 110,000 when crosslinked with a monomer-type OCIF; and (c) the protein has an activity to support and promote the differentiation and maturation of osteoclasts in a co-culture of mouse osteoblast-like stromal cells and mouse spleen cells in the presence of bone resorption factors such as the active-form of vitamin D_3 and parathyroid hormones (PTH).

As a representative *in vitro* culture system for osteoclastogenesis, a co-culture system of mouse-derived osteoblast-like stromal cell line, ST2, and mouse spleen cells in the presence of the active-form of vitamin D_3 or PTH is well known. The cells that express the protein of the present invention can be obtained by examining the binding ability of a mouse osteoblast-like stromal cell or mouse spleen cell cultured in the presence or absence of the active-form of vitamin D_3 to OCIF. The protein of the present invention is identified as a protein which is specifically induced on an osteoblast-like stromal cell cultured in the presence of bone resorption factors such as the active-form of vitamin D_3 or PTH. Further, in

consideration of the following facts that osteoclast formation is inhibited by addition of OCIF to the above co-culture system in the presence of the active-form of vitamin D_3, in a dose-dependent manner within a range of 1 to 40 ng/ml of OCIF; that there is an intimate correlation between change in expression of the present protein induced on the ST2 cells in the presence of the active-form of vitamin D_3 and the change in osteoclast formation with the passage of time; that the amount of the present protein expressed on a ST2 cell corresponds to the intensity of an ability to support osteoclast formation; and that osteoclast formation is completely inhibited by binding of OCIF(s) to the present protein on the ST2 cells, the protein of the present invention is identified as a protein having biological activity (effect) to support and promote the differentiation and maturation of osteoclasts.

The affinity of the protein of the present invention for OCIF can be assessed by labeling OCIF and testing the binding activity of the labeled OCIF to the surface of an animal cell membrane. OCIF can be labeled by a commonly used protein-labeling method such as labeling with a radioisotope or fluorescence labeling. For instance, an example of labeling OCIF with a radioisotope is ^{125}I labeling at a tyrosine residue, and labeling methods such as Iodogen method, chloramine T method and enzyme method can be employed thereto. The binding activity of the thus labeled OCIF to the surface of an animal cell membrane can be examined in accordance with a commonly used method, and the amount of nonspecific binding can be measured by adding 100 to 400 times excess amounts of unlabeled OCIF to the medium for the binding experiment. The amount of specific binding of OCIF is calculated by subtracting that of the nonspecific binding from that of total binding. The affinity (for OCIF) of the present protein expressed on a cell membrane is assessed by conducting the test with various amounts of the labeled OCIF and analyzing the amount of the specific binding by Scatchard plot. The determined affinity of the protein of the present invention for OCIF is about 100 to 500 pM. Thus, the protein of the present invention is identified as a protein having such high affinity (the dissociation constant, the Kd value, on a cell membrane is not larger than 10^{-9} M) for OCIF. The molecular weight of OBM is measured by use of gel filtration chromatography, SDS-PAGE or the like. To measure the molecular weight more accurately, SDS-PAGE is preferably used, and OBM is identified as a protein having a molecular weight of about 40,000 (40,000 ± 4,000) under reducing conditions.

The protein of the present invention can be obtained from a mouse osteoblast-like stromal cell line, ST2, a mouse fat cell strain PA6, or human osteoblast-like cell lines, or concentrated osteoblast-like cells obtained from mammals such as human, mouse and rat. And, substances that are required to express the protein of the present invention on these cells may be bone resorption factors such as the active-form of vitamin D_3 (calcitriol), parathyroid

hormone (PTH), interleukins (IL)-1, IL-6, IL-11, oncostatin M, and leukemia cell growth inhibiting factor (LIF). As for the amounts of these substances, it is desirable to use the active-form of vitamin D_3 or PTH in an amount of 10^{-8} M; the IL-11 and the oncostatin M in amounts of 10 ng/ml and 1 ng/ml, respectively; and the IL-6 in an amount of 20 ng/ml with 500 ng/ml of IL-6 soluble receptor. It is preferable to use cells obtained by culturing mouse osteoblast-like stromal cell line, ST2, in α-MEM containing 10^{-8} M of the active-form of vitamin D_3, 10^{-7} M dexamethasone and 10% bovine fetal serum for at least one week until the cells become confluent. Thus cultured cells can be removed and collected by using a cell scraper or the like. Moreover, the collected cells can be stored at -80°C until use.

The protein of the present invention can be purified efficiently from a membrane fraction of the thus collected cells. The membrane fraction can be prepared in accordance with a common method used for fractionation of organelles. As a buffer used in preparation of the membrane fraction, various protease inhibitors may be preferably added. Illustrative examples of protease inhibitors to be added include serine protease inhibitors, thiol protease inhibitors, and metalloprotease inhibitors, such as PMSF, APMSF, EDTA, O-phenanthroline, leupeptin, pepstatin A, aprotinin and a soybean trypsin inhibitor. To crush the cells, a Dounce homogenizer, a polythoron homogenizer, an ultrasonicator or the like can be used. The crushed cells can be suspended in a buffer containing 0.5 M sucrose and centrifuged at 600 X g for 10 minutes so as to separate cell nuclei and uncrushed cells as a precipitated fraction. After further centrifugation at 150,000 X g for 90 minutes, a membrane fraction can be obtained as a precipitated fraction. By treating the thus obtained membrane fraction with various surfactants, the protein of the present invention existing on the cell membrane can be solubilized and extracted, efficiently. For solubilization, various surfactants which are conventinally used in solubilization of cell membrane proteins, such as CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1- propanesulfonate), Triton X-100, Nikkol and n-octylglycoside, can be used. The protein of the present invention is preferably solubilized by adding 0.5% CHAPS to the protein and agitating the mixture at 4°C for 2 hours. By centrifuging the thus prepared sample at 150,000 X g for 60 minutes, the solubilized membrane fraction can be obtained as a supernatant.

The protein of the present invention can be purified efficiently from the thus obtained solubilized membrane fraction, using an OCIF-immobilized column, gel or resin. As the OCIF to be used in the immobilization, that isolated from the culture solution of human fetal lung fibroblasts, IMR-90, in accordance with a method described in WO 96/26217 or that obtained by genetic engineering (rOCIF) can be used. This rOCIF can be obtained by incorporating the corresponding human, rat or mouse cDNA into an expression vector in accordance with a common method, expressing the rOCIF with animal

or insect cells such as CHO cells, BHK cells and Namalwa cells, and then purifying it. The thus obtained OCIF shows a molecular weight of about 60 kDa (monomer type) and a molecular weight of about 120 kDa (dimer type). A dimer type OCIF is preferably used in the immobilization. As a gel or a resin for immobilizing OCIF, ECH SEPHAROSE® 4B, EAH SEPHAROSE® 4B, thiopropyl SEPHAROSE® 6B, CNBr-activated SEPHAROSE® 4B, activated CH SEPHAROSE® 4B, epoxy activated SEPHAROSE® 6B, activated thiol SEPHAROSE® 4B (products of Pharmacia Co., Ltd.), TSkgel AF-epoxy TOYOPAL 650, TSkgel AF-amino TOYOPAL 650, TSkgel AF-formyl TOYOPAL 650, TSkgel AF-carboxy TOYOPAL 650, TSkgel AF-Tresyl TOYOPAL 650 (products of Toso Co., Ltd.), amino-CELLULOFINE™ carboxy-CELLULOFINE™, FMP activated CELLULOFINE™, formyl-CELLULOFINE™ (products of Sei Kagaku Kougyo Co., Ltd.), AFFIGEL 10, AFFIGEL 15 and AFFIPREP 10 (products of Bio-Rad Co., Ltd.) are available. Furthermore, as a column for immobilizing OCIF, a HITRAP® NHS-activated column (Pharmacia Co., Ltd.), TSKgel Tresyl-5PW (Toso Co., Ltd.) or the like can be used. As a specific example of a method for immobilizing OCIF with the HITRAP® NHS-activated column (1 ml, Pharmacia Co., Ltd.), the following method is presented. That is, 1 ml of 0.2 M $NaHCO_3$/0.5 M NaCl (pH 8.3) solution containing 13.0 mg of OCIF is applied to the column and allowed to undergo a coupling reaction at room temperature for 30 minutes. Then, after 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) and 0.1 M acetic acid/0.5 M NaCl (pH 4.0) are applied, respectively, 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) is applied again, and then the column is left to stand at room temperature for 1 hour so as to inactivate excess active groups. Thereafter, the column is washed twice with 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) and 0.1 M acetic acid/0.5 M NaCl (pH 4.0), and then replaced with 50 mM Tris/1 M NaCl/0.1% CHAPS buffer (pH 7.5). Finally, an OCIF-immobilized column can be prepared. Using the prepared OCIF-immobilized column, gel or resin, the protein of the present invention can be purified efficiently. To prevent the proteolysis of the protein of the present invention, the above various protease inhibitors may also be added to the buffer solution used in purification. After applying the above solubilized membrane fraction to an OCIF-immobilized column or mixing the solubilized membrane fraction with an OCIF-immobilized gel or resin and subsequently stirring the mixture so as to cause the fraction to be adsorbed, the protein of the present invention can be eluted from the OCIF-immobilized column, gel or resin using an acid, various protein-denaturating agents, a cacodylate buffer or the like. To minimize denaturation of the protein of the present invention, it is preferable to neutralize the eluate immediately using a base. As an acid buffer solution used for elution, 0.1 M glycine-hydrochloric acid buffer solution (pH 3.0), 0.1 M glycine-hydrochloric acid buffer solution (pH 2.0) and 0.1 M sodium citrate buffer solution (pH 2.0) can be used, for example.

The purified protein of the present invention can be further purified by use of a method which is conventionally employed in purification of proteins from biological samples, through various purification operations taking advantages of the physicochemical properties of the protein of the present invention. To concentrate a solution of the protein of the present invention, a method which is conventionally used in protein purification process, e.g., ultrafiltration, freeze-drying and salting-out, can be used. Preferably, ultrafiltration based on centrifugation with CENTRICON®-10 (Amicon Co.) and the like is used. Furthermore, as a means of purificiation, various methods conventionally used in protein purification using ion exchange chromatography, gel filtration chromatography, hydrophobic chromatography, reversed phase chromatography, preparative electrophoresis and the like can be used in combination. More specifically, the protein of the present invention can be purified by concombined use of gel filtration chromatography with SUPEROSE®-12 column (Pharmacia Co., Ltd.) and the like and reverse phase chromatography. Moreover, the protein of the present invention during the purification process can be detected by analyzing activity to bind the immobilized OCIF or by immuno precipitation of OCIF-binding substances with an anti-OCIF antibody followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

The thus obtained protein of the present invention is useful, due to its activity, as medicaments, e.g., as agents for treating bone metabolism abnormality such as osteopetrosis, or experimental and diagnostic reagents.

Furthermore, the present invention relates to DNA which encodes a novel protein (OCIF binding molecule; OBM) which binds osteoclastogenesis inhibitory factor (OCIF), a protein having an amino acid sequence encoded by the DNA, a method for genetically producing a protein which specifically binds OCIF by use of the protein, and an agent for treating bone metabolism abnormality comprising the protein. In addition, the present invention relates to a method for screening a substance which controls expression of OBM, a method for screening a substance which binds OBM and inhibits or modifies an effect thereof, a method for screening a receptor which binds OBM and transmits an effect thereof, and pharmaceutical compositions comprising a substance obtained as a result of these methods for screening.

The novel protein OBM encoded by the DNA of the present invention shows the following physicochemical properties and biological activity. That is, (a) the protein specifically binds osteoclastogenesis inhibitory factor (OCIF), (b) the protein shows a molecular weight of about 40,000 (± 4,000) as measured by SDS-PAGE under reducing conditions, and shows an apparent molecular weight of about 90,000 to 110,000 when crosslinked with a monomer-type OCIF; and (c) the protein has an activity to support and

promote differentiation and maturation of osteoclasts.

Human osteoclastogenesis inhibitory factor (OCIF) is used as a probe for assessing the properties of OBM in identification of the DNA encoding OCIF binding molecule OBM of the present invention, and can be isolated from the culture solution of human fetal lung fibroblasts, IMR-90, in accordance with WO 96/26217. For isolation and identification of the DNA encoding OBM, recombinant human OCIF, recombinant mouse OCIF, recombinant rat OCIF and the like can also be used. This recombinant OCIF can be obtained by incorporating the corresponding DNA into an expression vector in accordance with a commonly used method, subsequently expressing OCIF with animal or insect cells such as CHO cells, BHK cells and Namalwa cells, and then purifying it.

Methods for cloning a cDNA which encodes the target protein (cDNA cloning) include, a method comprising the steps of determining a partial amino acid sequence of the protein and isolating the target cDNA by hybridization based on a nucleotide sequence corresponding to the amino acid sequence. Another method comprises the steps of constructing a cDNA library with an expression vector, regardless of whether or not the amino acid sequence of the protein is known, subsequently introducing it into cells, and then screening for the presence and absence of expression of the target protein and isolating the desired cDNA (D' Andrea *et al.*: Cell 57, 277 to 285, 1989; Fukunaga *et al.*: Cell 61, 341 to 350, 1990) (expression cloning method). In the expression cloning method, bacterial, yeast, animal cells and the like are selected and used as host cells according to the purpose. For cloning a cDNA which encodes a protein considered to present on the surface of animal cell membrane as in the present invention, animal cells are often used as hosts. Furthermore, hosts with high efficiency for introducing DNA and expressing the introduced DNA are conventionally used. One of the cells having such characteristics is a monkey kidney cell line, COS-7, used in the present invention. Since SV40 large T antigen is expressed in COS-7 cells, plasmids having a SV40 origin of replication are present in the cell as multicopy episome, whereby higher expression than usual can be expected. Moreover, since the maximum expression level is reached within a few days after the introduction of DNA, COS-7 cells are suitable for quick screening. In combination with a plasmid suitable for high expression, this host cell enables an extremely high level of gene expression. The promoter is a factor of a plasmid which has the most significant effects on the amount of gene expression. As a promoter suitable for high level of expression, SRα promoter and cytomegalovirus-derived promoter are often used. Screening methods for cloning the cDNA of the membrane protein include expression cloning, binding method, panning method and film emulsion method.

The present invention relates to DNA, which encodes the protein which

specifically binds OCIF (OBM), obtained by a combination of the expression cloning method and the binding method, the protein expressed therewith, and a screening of a biologically active substance with the DNA or the protein. OBM encoded by the DNA of the present invention can be detected by labeling OCIF and subsequently examining the binding activity of the labeled OCIF to the surface of an animal cell membrane. OCIF can be labeled by a conventional method for labeling protein such as labeling with a radioisotope or fluorescence labeling. An example of labeling OCIF with radioisotope is ^{125}I labeling at tyrosine residues, and specific labeling methods include Iodogen method, chloramine T method and enzyme method. The binding activity of thus labeled OCIF to the surface of an animal cell membrane can be assessed in accordance with a commonly used method. Furthermore, an amount of nonspecific binding can be measured by adding 100 to 400 times excess amount of unlabeled OCIF to the medium for a binding experiment. The amount of specific binding of OCIF is calculated by subtracting that of the nonspecific binding from that of the total binding.

Based on an assumption that a factor, which is involved in differentiation of osteoclasts, interacts with OCIF, the inventors have screened an expression library prepared from the mRNA of a mouse osteoblast-like stromal cell line, ST2, with recombinant OCIF in accordance with the following method in order to separate the protein which binds OCIF. DNA synthesized from the mRNA of ST2 cells was inserted into an expression vector for an animal cell, and they were transduced (transfected) into COS-7 monkey kidney cells. Using ^{125}I-labeled OCIF as a probe, the target protein expressed on the COS-7 cell was screened. As a result, DNA which encodes the protein that specifically binds OCIF could be separated, and then the nucleotide sequence of the DNA which encodes this OCIF binding molecule (OCIF binding molecule; OBM) was determined. Furthermore, it has been found that OBM encoded by the DNA strongly and specifically binds OCIF on the cell membrane.

An example of DNA hybridization under relatively mild conditions in the present invention is that after DNA is transferred to a nylon membrane and fixed in accordance with a common method, it is hybridized with a radio-labeled DNA as a probe in a hybridization buffer at 40 to 70°C for about 2 hours to overnight, and then washed with 0.5 X SSC (0.075 M sodium chloride and 0.0075 M sodium citrate) at 45°C for 10 minutes. More specifically, after DNA is transferred and fixed to a nylon membrane, HYBOND® N (Amersham Co., Ltd.), in accordance with a conventional method, it is hybridized with a ^{32}P-labeled DNA as a probe in Rapid Hybridization Buffer (Amersham Co., Ltd.) at 65°C for 2 hours, and then washed with 0.5 X SSC (0.075 M sodium chloride and 0.0075 M sodium citrate) at 45°C for 10 minutes.

As a representative *in vitro* culture system for osteoclastogenesis, a co-culture system of mouse-derived osteoblast-like stromal cell line, ST2, and mouse spleen cells in the

presence of the active-form of vitamin D_3 or PTH is well known. OBM of the present invention is identified as a protein which is specifically induced on an osteoblast-like stroma cell cultured in the presence of bone resorption factors such as an active-form of vitamin D_3 and PTH. Furthermore, since osteoclasts formation is stimulated by adding the protein encoded by the DNA of the present invention to a culture system of mouse spleen cells even in the absence of the active-form of vitamin D_3 or PTH, OBM encoded by the DNA of the present invention is considered to be involved in differentiation and maturation of osteoclasts.

A recombinant OBM can be produced by inserting the DNA of the present invention into an expression vector so as to prepare a plasmid for expressing OBM, and then introducing and expressing the plasmid in various cells and microbial strains. COS-7, CHO, Namalwa and the like can be used as mammalian hosts cells for expression, and *Escherichia coli (E. coli)* and the like can be used as bacterial host cells for expression. In such a case, the recombinant OBM can be expressed as a membrane-bound protein using full length of DNA or as a secretory-type or solubilized-type (soluble-type) protein by removing a part of the DNA encoding a membrane-binding domain from the full length. Thus produced recombinant OBM can be purified efficiently in combination with conventional methods used in protein purification such as affinity chromatography using an OCIF-immobilized column, ion exchange chromatography, gel filtration chromatography and the like. The thus obtained protein of the present invention is useful, due to its activity, as medicaments, e.g., as agents for treating bone metabolism abnormality such as osteopetrosis and as experimental or diagnostic reagents.

The protein OBM encoded by the DNA of the present invention enables: (1) screening of a substance which controls expression of OBM; (2) screening of a substance which specifically binds OBM and inhibits or modifies the biological activity of OBM; and (3) screening of a protein (OBM receptor) which exists on a precursor cell of osteoclasts and transmits the biological activity of OBM; and (4) as well as developments of antagonists and agonists using this OBM receptor. In combinatorial chemistry using the above OBM or OBM receptor, a peptide library required to identify an antagonist or agonist can be prepared in accordance with the following specific methods. One of them is a split method (Lam *et al.*; Nature 354, 82 to 84, 1991). In this method, synthetic carriers (beads) are bound to amino acids (units), separately. Then, these synthetic beads are mixed together and divided into an equal number of units, and then bound to the subsequent units. By repeating this operation n times, a library in which n units are bound to the carriers is prepared. Such an operation allows the synthesis of only one sequence per one group of the carriers. Hence, when a positive carrier group is selected in said method for screening by use of the protein of

the present invention and then the amino acid sequence thereof is determined, a specifically binding peptide can be identified. As another method, a phage display method can be used. In this method, synthetic genes encoding random peptides are expressed using phage. While this method has an advantage that it can archive a larger number of molecules in a library than the above synthetic library, it also has a disadvantage that the kind of peptides per molecules is not as varied because peptides having sequences that phages don't prefer do not exsist in the library. In the phage display method, as in the case of the split method, using a screening system with the protein of the present invention, phage specifically binding thereto are concentrated by panning. Thus obtained phage are amplified in *E. coli*, and further, the nucleotide sequence encoding the peptide is determined. Furthermore, when it is desired that a specific peptide having high affinity for OBM or OBM receptor is screened from a peptide library using the screening system of the above (2) or (3), a specific peptide having a very high affinity can be obtained by screening a positive carrier or phage in the co-presence of OCIF or OBM with a change of concentration. For example, screening of a peptide agonist of low molecular weight having an EPO-like activity from a varied peptide library with an erythropoietin (EPO which is a hematopoietic hormone) receptor, analysis of a three-dimensional structure thereof, and the production of a substance (agonist) of low-molecular-weight having an EPO activity through synthesis of organic chemical compounds based on the three-dimensional structure has already been successful (Nicholas *et al.*: Science, 273, 458 to 463, 1996).

Furthermore, the inventors have found that a protein binding OCIF is specifically expressed on an osteoblast-like stromal cell line, ST2, which was cultured in the presence of bone resorption factors such as the active-form of vitamin D_3 and parathyroid hormone (PTH), using osteoclastogenesis inhibitory factor (OCIF). Moreover, the inventors have found that the protein, which is associated with differentiation of immature osteoclast precursor cells to osteoclasts and maturation thereof, has a biological activity as a factor which supports and promotes so-called differentiation and maturation of osteoclasts. After purification of the protein, the physicochemical properties and biological activity of the protein were examined. The inventors have compared the physicochemical properties and biological activity of the recombinant protein OBM by expressing the DNA of the present invention with those of a purified natural-type protein which specifically binds OCIF in order to clarify differences between them. As a result, they have found that (1) each of both proteins is a membrane-bound protein and specifically binds OCIF; (2) they shared a molecular weight of about 40,000 as measured by SDS-PAGE; and (3) they have an apparent molecular weight of about 90,000 to 110,000 when crosslinked with a monomer-type OCIF, which indicates that they have very similar physicochemical properties. An activity to

support and promote differentiation and maturation of osteoclasts was also shared by them as well. Therefore, the possibility that both proteins are identical was suggested. Furthermore, an anti-OBM rabbit polyclonal antibody prepared with the protein (recombinant OBM), which was genetically expressed with the DNA of the present invention and then purified, has cross-reactivity to the purified natural-type protein obtained by the above method and specifically inhibited the binding between said natural-type protein and OCIF, just as it inhibits specific binding between OBM and OCIF. From these results, it is obvious that the recombinant protein OBM expressed with the DNA of the present is identical to the natural-type protein which specifically binds OCIF.

Furthermore, for isolating a gene (cDNA) that encodes a human-derived OCIF binding protein molecule (hereinafter referred to as "human OBM") which specifically binds OCIF and has an activity to support and promote differentiation of mouse spleen cells to osteoclasts and maturation, just as the natural-type or the recombinant mouse OBM does, the inventors have carried out a polymerase chain reaction (PCR) using primers prepared based on the above mouse OBM cDNA and human lymph node-derived cDNA as a template. Thus, the inventors have screened said cDNA library with the obtained human OBM cDNA fragment. As a result, they have succeeded in isolation of the cDNA which encodes the human-derived protein which specifically binds OCIF (human OBM) and determination of the nucleotide sequence of said cDNA. They have found that human OBM encoded by the cDNA strongly and specifically binds OCIF on a cell membrane and has a biological activity to support and promote differentiation of mouse spleen cells to osteoclasts and maturation thereof, just as mouse OBM does. That is, other objects of the present invention are to provide: (1) DNA which encodes human OBM which is a novel human-derived protein which binds osteoclastogenesis inhibitory factor (OCIF; a protein having an amino acid sequence encoded by the DNA) (2) a method for genetically producing a protein which specifically binds OCIF and has an activity to support and promote differentiation of mouse spleen cells to osteoclasts and maturation thereof by use of the DNA; (3) an agent for treating bone metabolism abnormality comprising the protein; (4) a method for screening a substance which controls expression of human OBM; (5) a method for screening a substance which binds human OBM and inhibits or modifies an effect thereof; (6) a method for screening a receptor which binds human OBM and transmits an effect thereof; and (7) a pharmaceutical composition comprising a substance obtained as a result of these methods for screening.

The present invention relates to DNA which encodes human OBM, a novel human protein, which specifically binds OCIF and has a biological activity to support and promote differentiation and maturation of osteoclasts; a protein having an amino acid sequence encoded by the DNA; a method for genetically producing a protein which

specifically binds OCIF and has an activity to support and promote differentiation and maturation of osteoclasts with the DNA; and an agent for treating bone metabolism abnormality comprising the protein. Furthermore, the present invention also relates to a method for screening a substance which controls expression of human OBM; a method for screening a substance which binds human OBM and inhibits or modifies an effect thereof; a method for screening a receptor which binds human OBM and transmits a biological activity of OBM; a pharmaceutical composition comprising a substance obtained as a result of these methods for screening; an antibody to the human-derived OCIF binding protein; and an agent for preventing and/or treating bone metabolism abnormality using the antibody.

The novel human-derived OCIF binding protein molecule, human OBM, encoded by the DNA of the present invention shows the following physicochemical properties and biological activity. That is, (a) human OBM specifically binds to osteoclastogenesis inhibitory factor (OCIF) (WO 96/26217); (b) human OBM shows a molecular weight of about 40,000 (± 5,000) as measured by SDS-PAGE under reducing conditions and shows an apparent molecular weight of about 90,000 to 110,000 when crosslinked with a monomer-type OCIF; and (c) human OBM has a biological activity to support and promote differentiation and maturation of osteoclasts.

The cDNA encoding mouse OBM, mouse-derived OCIF binding protein, useful as a probe for separating and identifying the cDNA which encodes human OBM of the present invention, can be isolated from a cDNA library of a mouse osteoblast-like stromal cell line, ST2. Furthermore, human osteoclastogenesis inhibitory factor (OCIF), required to examine the properties and biological activity of the protein obtained by expressing human OBM cDNA, can be isolated from the culture solution of human fibroblast strain IMR-90 in accordance with the method described in WO 96/26217, or it can be genetically produced with the DNA encoding it. To examine the properties and biological activity of human OBM, recombinant humOCIF, recombinant mouse OCIF, recombinant rat OCIF and the like can also be used. These recombinant OCIFs can be obtained by incorporating the corresponding cDNAs into expression vectors in accordance with a commonly used method, expressing OCIFs in animal or insect cells such as CHO cells, BHK cells and Namalwa cells, and purifying them.

Methods for isolating the human cDNA which encodes the target protein (cDNA cloning) include: (1) a method comprising the steps of purifying the protein, determining a partial amino acid sequence thereof, and isolating the target cDNA by a hybridization with DNA having a nucleotide sequence corresponding to the amino acid sequence as a probe, (2) a method (expression cloning method) comprising the steps of constructing a cDNA library with an expression vector, regardless of whether the amino acid

sequence of the target protein is unknown, introducing them into cells, and screening for the presence and absence of the expression of the target protein so as to isolate the target cDNA; and (3) a method of isolating the cDNA which encodes the target human protein by the hybridization or polymerase chain reaction (PCR) method from cDNA library constructed from human cells or tissue or using cDNA which encodes a protein derived from a mammal other than human and having the same properties and biological activity of the human-derived target protein as a probe, based on an assumption that the cDNA which encodes the non-human protein shares high homology with that which encodes the desired corresponding human protein to be cloned.

Based on an assumption that human OBM cDNA is highly homalogous with the above mouse OBM cDNA, human cells or tissue producing human OBM can be identified by Northern hybridization method using the latter (mouse) cDNA as a probe. Human OBM cDNA can be cloned as follows. A human OBM cDNA fragment is obtained through PCR using mouse OBM primers prepared based on the mouse OBM cDNA and the cDNA library of a cell or tissue which produces human OBM (e.g., a human lymph node) as identified above, as primers and a template, respectively. The cDNA library of cells or tissue which produce human OBM as identified above is screened with the human OBM cDNA fragment as a probe, and thus, human OBM cDNA can be obtained. The present invention relates to the obtained DNA that encodes human OBM, a human-derived protein which specifically binds OCIF and has biological activity to support and promote differentiation and maturation of osteoclasts. Since human OBM encoded by the DNA of the present invention is a mambrane-bound protein having a transmembrane domain, it can be detected by labeling OCIF and then binding the labeled OCIF to the surface of an animal cell in which the cDNA of the present invention is expressed. In such a case, OCIF can be labeled by a method which is conventinally used for labeling protein such as labeling with a radioisotope and fluorescence labeling.

The molecular weight of the protein expressed by human OBM cDNA of the present invention is determined by gel filtration chromatography, SDS-PAGE and the like. To determine the molecular weight more accurately, SDS-PAGE is preferably used, and human OBM is identified as a protein having a molecular weight of about 40,000 (40,000 ± 5,000) under reducing conditions.

An example of DNA hybridization under relatively mild conditions in the present invention is that after DNA is transferred to a nylon membrane and fixed in accordance with a commonly used method, the DNA is hybridized with another radiolabeled DNA as a probe in a hybridization buffer at 40° to 70°C for about 2 hours to overnight and then washed with 0.5 X SSC (0.075 M sodium chloride and 0.0075 M sodium citrate) at 45°C

for 10 minutes. More specifically, after DNA is transferred and fixed to a nylon membrane, which is HYBOND® N (Amersham Co., Ltd.), in accordance with a conventional method, the DNA is hybridized with another ^{32}P-labeled DNA as a probe in Rapid Hybridization Buffer (Amersham Co., Ltd.) at 65°C for 2 hours, and then washed with the above 0.5X SSC at 45°C for 10 minutes.

As a representative *in vitro* culture system for osteoclastogenesis, a co-culture system of mouse-derived osteoblast-like stromal cell line, ST2, and mouse spleen cells in the presence of the active-form of vitamin D_3 or PTH is well known. For promoting osteoclastogenesis in this *in vitro* culture system, both the interaction between a osteoblast-like stromal cell and a spleen cell through their binding, and the presence of bone resorption factors such as the active-form of vitamin D_3 and PTH are essential. In this *in vitro* culture system, a recombinant COS cell strain, resulting from the expression of the cDNA of the present invention thereon, has obtained an ability to support osteoclast formation from spleen cells, just like the osteoblast-like stromal cell line ST2, while COS-7 cells (a monkey kidney-derived cell line) does not have an ability to support osteoclast formation in the absence of said bone resorption factors. Furthermore, since the cDNA of the present invention encodes a membrane-bound protein, the protein can be expressed as a secretory-type or solubilized-type protein after removing the fragment which encodes the membrane binding domain thereof. It has also been confirmed that osteoclastogenesis was promoted simply by adding the secretory-type human OBM to the above *in vitro* culture system in the absence of said bone resorption factors. From these results, human OBM encoded by the cDNA of the present invention is identified as a factor involved in differentiation and maturation of osteoclasts.

Recombinant human OBM can be produced by inserting the cDNA of the present invention into an expression vector so as to prepare a plasmid for expressing human OBM and then introducing and expressing the plasmid in various cells and strains. COS-7, CHO and Namalwa cells and the like can be used as mammalian host cells suitable for expression, and *E. coli* and the like can be used as bacterial host cells. In those cases, recombinant human OBM can be expressed as a membrane-bound protein by using the full length of DNA or as a secretory-type or solubilized-type protein by removing a region which encodes the membrane binding domain. Thus produced recombinant human OBM can be purified efficiently in combination wiht conventionally used methods for purifying protein such as affinity chromatography using OCIF-immobilized or a column, ion exchange chromatography, gel filtration chromatography and the like. Thus obtained human OBM of the present invention is useful, due to its activity, as a medicament, e.g., as an agent for treating bone metabolism abnormality such as osteopetrosis or as an experimental and

diagnostic reagent.

The human OBM protein encoded by the cDNA of the present invention enables: (1) screening of a substance which controls expression of human OBM; (2) screening of a substance which specifically binds human OBM and inhibits or modifies the biological activity of human OBM; and (3) screening of a human protein (human OBM receptor) which exists in a precursor cell of human osteoclasts and transmits the biological activity of human OBM, as well as development of antagonist and agonist using this human OBM receptor. In combinatorial chemistry using the above human OBM or human OBM receptor, a peptide library, which is employed for identification of an antagonist or agonist, can be prepared in accordance with the same method using the mouse OBM. After screening the peptide library by said method in which human OBM is used instead of mouse OBM, a specific peptide having very high affinity can be obtained.

Furthermore, for measurement of OBM, a highly useful protein described above, it is necessary to obtain an antibody which specifically recognizes OBM and establish an enzyme immunoassay using it. However, no antibody useful for measurement of OBM has been available. Moreover, an anti-OBM/sOBM antibody which neutralizes the biological activity of OBM or sOBM is assumed to suppress an activity of OBM or sOBM to promote osteoclast formation, and expected to be developed as an agent for treating bone metabolism abnormality. However, such an antibody has not been available.

Under that circumstance, the inventors have made intensive studies and have found antibodies (anti-OBM/sOBM antibodies) which recognize both of the following antigens, a membrane-bound protein (OCIF binding molecule; OBM) which specifically binds osteoclastogenesis inhibitory factor (OCIF) and a soluble-type OBM (sOBM) lacking the membrane binding domain. Therefore, objects of the present invention are to provide: (1) an antibody (anti-OBM/sOBM antibody) which recognizes both of the following antigens, a membrane-bound protein (OBM) which specifically binds to osteoclastogenesis inhibitory factor (OCIF) and soluble OBM (sOBM) lacking the membrane binding domain; (2) a method for production thereof; (3) a method for measuring OBM and the sOBM by use of said antibody; and (4) an agent for preventing and/or treating bone metabolism abnormality which comprises said antibody as an active ingredient.

The present invention relates to: (1) an antibody (anti-OBM/sOBM antibody) which recognizes both of the following antigens, a membrane-bound protein (OCIF binding molecule; OBM) which specifically binds osteoclastogenesis inhibitory factor (OCIF) and a soluble-type OBM (sOBM) lacking the membrane binding domain; (2) a method for production thereof; (3) a method for measuring OBM and the sOBM by use of said antibody; and (4) a pharmaceutical compisition comprising said antibody as an active ingredient,

particularly, an agent for preventing and/or treating bone metabolism abnormality.

An antibody of the present invention is an antibody which has an activity to neutralize osteoclastogenesis promoting activity, which is a biological activity that OBM and sOBM have, said antibody has any of the following properties: a) a polyclonal antibody which recognizes both mouse OBM and mouse sOBM antigens (anti-mouse OBM/sOBM polyclonal antibody); b) a polyclonal antibody which recognizes both human OBM and human sOBM antigens (anti-human OBM/sOBM polyclonal antibody); c) a monoclonal antibody which recognizes both mouse OBM and mouse sOBM antigens (anti-mouse OBM/sOBM polyclonal antibody); d) a monoclonal antibody which recognizes both human OBM and human sOBM antigens (anti-human OBM/sOBM polyclonal antibody); and e) an anti-human OBM/sOBM monoclonal antibody which has crossreactivity to both mouse OBM and mouse sOBM antigens.

The polyclonal antibody which recognizes both mouse OBM and mouse sOBM antigens (hereinafter referred to as "anti-mouse OBM/sOBM polyclonal antibody") and the polyclonal antibody which recognizes both human OBM and human sOBM antigens (hereinafter referred to as "anti-human OBM/sOBM polyclonal antibody") can be obtained by the following means. A purified mouse OBM as an antigen for immunization can be obtained in accordance with the above method. That is, natural-type mouse OBM can be obtained by treating a mouse osteoblast-like stromal cell line, ST2, with the active-form of vitamin D_3 and subsequently purifying it from the cell membranes of said cell by means of OCIF immobilized on a column and gel filtration chromatography. Alternatively, after incorporating the above mouse OBM cDNA (SEQ ID NO: 15) or human OBM cDNA (SEQ ID NO: 12) into an expression vector, expressing OBM in an animal or insect cell such as a CHO cell, a BHK cell, Namalwa or a COS-7 cell or *E. coli* and then purifying by the same method as described above, recombinant mouse OBM (SEQ ID NO: 1) or recombinant human OBM (SEQ ID NO: 11) can be obtained, and these may also be used as antigens for immunization. At this time, it takes tremendous effort to highly purify a large quantity of mouse OBM or human OBM, a membrane-bound protein (OBM). On the other hand, it has been confirmed that there is no difference in ability to promote differentiation and maturation of osteoclasts between OBM, a membrane-bound protein, and soluble-type OBM (sOBM), which is a soluable protein obtained by deleting the membrane binding domain of OBM as described above. Accordingly, taking into account that expression and high purification of mouse sOBM and human sOBM are relatively easy, these sOBMs, solubilized proteins, may be used as antigens for immunization. Mouse sOBM (SEQ ID NO: 16) and human sOBM (SEQ ID NO: 17) can be obtained by adding a nucleotide sequence, which encodes a known signal sequence derived from other secretory-type proteins, 5' upstream of the mouse sOBM

cDNA (SEQ ID NO: 18) or the human sOBM cDNA (SEQ ID NO: 19), incorporating the cDNA into an expression vector in accordance with the same gene engineering method as described above, expressing the protein in a variety of animal cells, insect cells or *E. coli* as a host, and then purifying. Thus obtained antigen for immunization is dissolved in a phosphate buffered saline solution (PBS) and, if necessary, mixed with an equal volume of Freund's complete adjuvant and emulsified. Then, an animal is immunized with the emulsion through a few times of subcutaneous administration with a one-week interval between each. The antibody titer is measured. When the value reaches maximum, booster administration is performed. On the 10^{th} day from the booster administration, all the blood was collected. The obtained antiserum is fractionated and precipitated with ammonium sulfate, and the globulin fraction is purified with an anion exchange chromatography or the antiserum is diluted twice with Binding Buffer (Bio-Rad Co., Ltd.) and the diluted antiserum is purified by Protein A or Protein G SEPHAROSE® (Pharmacia Co., Ltd.) column chromatography. Thereby, the desired anti-mouse or anti-human OBM/sOBM polyclonal antibody can be obtained.

The monoclonal antibody of the present invention can be obtained by the following method. That is, as an antigen for immunization required to prepare the monoclonal antibody, a natural-type mouse OBM, recombinant mouse or human OBM, or recombinant mouse or human sOBM can be used, as used in preparation of the above polyclonal antibody. Lymphocytes derived from immunized mammals with each antigen or that obtained by *in vitro* methods are fused with a myeloma cell line, and hybridomas are prepared in accordance with a conventional method. From the culture of this hybridoma, a hybridoma producing an antibody which recognizes each antigen is selected by solid-phase ELISA, using each highly purified antigen. The obtained hybridoma is cloned, and a thus obtained stable antibody-producing hybridoma is cultured. The target antibody can be obtained therefrom. For preparation of the hybridoma, immunizing a mammal, a small animal such as a mouse or rat, is commonly used. To immunize the animal, a method comprising the following steps is conventinally used: diluting the antigen with an appropriate solvent such as physiological saline solution to an appropriate concentration and then administering the solution and, if necessary, co-administering Freund's complete adjuvant, into vein (i.v.) or the abdominal cavity (i.p.), about 3 or 4 times in total with a 1 to 2-week interval between each. The immunized animal is dissected on the 3^{rd} day after the last immunization, and spleen cells are obtained from the isolated spleen and used as immunocytes (immunized cells). Illustrative examples of mouse-derived myeloma for cell fusion with the immunocytes include p3/x63-Ag8, p3-U1, NS-1, MPC-11, SP-2/0, F0, P3x63 Ag8. 653 and S194. Furthermore, illustrative examples of rat-derived cells include cell lines such as R-210. For producing human antibody, human B lymphocyte cells are immunized *in*

vitro and fused with human myeloma cells or a cell line transformed by EB virus. Fusion of an immunized cell with a myeloma cell line is performed according to a known methods such as that of Koehler and Milstein *et al.*(Koehler *et al.*: Nature 256, 495 to 497, 1975), while an electric pulse method using an electric pulse may also be used. Immunized lymphocyte cells and myeloma cell lines are mixed together at a ratio conventionally used and fused in common bovine fetal serum (FCS)-free medium for cell culture in which polyethylene glycol is added. Then, culture is carried out in FCS-containing HAT selective medium so as to select a fused cell (hybridoma). Then, a hybridoma producing an antibody is selected by a commonly used method for detecting antibody such as ELISA, plaque method, ouchterlony method or condensation method. Thereafter, a hybridoma is established. The established hybridoma can be subcultured by a common method for culture and can be stored in a frozen state if necessary. The hybridoma may be cultured in accordance with a conventionally used method or transplanted in the abdominal cavity of the mammal. The antibody can be collected from the resulting culture solution or ascites, respectively. The antibody in the culture solution or ascites can be purified by a commonly used method such as a salting-out method, ion exchange chromatography, gel filtration chromatography, or Protein A or Protein G affinity chromatography. Almost all the monoclonal antibodies obtained by the above-described method using sOBM as an antigen are antibodies which can specifically recognize not only sOBM but also OBM (hereinafter referred to as "anti-OBM/sOBM monoclonal antibody"). These antibodies can be used for measurements of OBM and sOBM. After these antibodies are labeled with a radioisotope or an enzyme and thus employed to measurement systems known such as as radioimmunoassay (RIA) and enzyme immunoassay (EIA), an amount of OBM and sOBM can be measured thereby. By use of these measurement systems, an amount of sOBM in a living sample such as blood or urine can be measured with ease and with high sensitivity. Furthermore, by use of these antibodies, an amount of OBM bound to the surface of a tissue or cell can be measured through a binding assay or the like with ease and with high sensitivity.

When the obtained antibody is used as a medicament for humans, it is desirable, in consideration of a problem of antigenicity, that a human-type anti-human OBM/sOBM antibody is prepared. The human-type anti-human OBM/sOBM antibody can be prepared by the following methods. That is, (1) human lymphocyte cells extracted from human peripheral blood or the spleen are sensitized *in vitro* with human OBM or human sOBM as an antigen in the presence of IL-4, and then the sensitized human lymphocyte cells are fused with K_6H_6/B_5 (ATCC CRL1823), which is a hetero-hybridoma of mouse and human, thereby, a hybridoma producing the desired antibody is screened. An antibody produced from the obtained hybridoma is a human-type anti-human OBM/sOBM monoclonal antibody. Among these

antibodies, an antibody which neutralizes the activity of human OBM/sOBM is selected. However, it is usually difficult to obtain an antibody having high affinity for an antigen through such a method of sensitizing human lymphocyte cells *in vitro*. Therefore, for obtaining a monoclonal antibody having high affinity for human OBM and sOBM, it is necessary to modify an anti-human OBM/sOBM monoclonal antibody with low affinity as described above to be that with high affinity. A random mutation is introduced into a CDR region (CDR-3 in particular) of said human-type anti-human OBM/sOBM monoclonal antibody with low affinity which a neutralizing antibody obtained as described above. This is expressed with phage. Phage which strongly bind human OBM/sOBM as the antigen are selected by phage display method using a plate in which human OBM/sOBM is immobilized. The phage is allowed to proliferate in *E. coli*, and the deduced amino acid sequence of the CDR having high affinity is determined based on the nucleotide sequence thereof. The obtained gene which encodes the human-type anti-human OBM/sOBM monoclonal antibody is incorporated and expressed in a conventionally used expression vector for mammalian cells, and then human-type anti-human OBM/sOBM monoclonal antibodies can be obtained. Among them, the desired human-type anti-human OBM/sOBM monoclonal antibody which neutralizes the biological activity of human OBM/sOBM and has high affinity thereto can be selected. Furthermore, (2) using a Balb/c mouse, an anti-human OBM/sOBM mouse monoclonal antibody is prepared according to a conventinally used method (Koehler *et al.*, Nature 256, 495 to 497, 1975) as in the present invention, and a monoclonal antibody which neutralizes the biological activity of human OBM/sOBM and has high affinity thereto is selected. By CDR grafting method (Winter and Milstein: Nature 349, 293 to 299, 1991), that is a method in which a CDR region (CDR-1, 2 and 3) of the anti-human OBM/sOBM mouse monoclonal antibody with high affinity are transplanted into the CDR regions of human IgG, a humanized antibody can be obtained. Moreover, (3) human peripheral blood lymphocyte cells are transplanted into a severe combined immune deficiency (SCID) mouse. Thus transplanted SCID mouse produces a human antibody (Mosier D. E. *et al.*: Nature 335, 256 to 259, 1988: Duchosal M. A. *et al.*: Nature 355, 258 to 262, 1992). The cells are sensitized with human OBM or sOBM as an antigen and screened. Thereafter, a lymphocyte cell which produces a human-type monoclonal antibody specific to human OBM/sOBM can be extracted from the mouse. Then, as in the case of the above method for preparing a human-type antibody (1), the obtained lymphocyte cells are fused with K_6H_6/B_5 (ATCC CRL1823), a hetero hybridoma of mouse and human, and then the obtained hybridomas are screened. Then, a hybridoma which produces the target human-type monoclonal antibody can be obtained. By culturing the thus obtained hybridoma, the target human-type monoclonal antibody can be produced in large quantities. After purifying them in the same manner as

described above, large amouts of pure products thereof can be obtained. Furthermore, a recombinant human-type monoclonal antibody can be produced in large quantities by constructing a cDNA library from said hybridoma which produces the target human-type monoclonal antibody, cloning the cDNA which encodes the target human-type monoclonal antibody, incorporating said cDNA into an appropriate expression vector by gene engeneering, and expressing the antibody in a variety of animal cells, insect cells or *E. coli* as a host. After purifaction of the antibody from said culture according to the method as described above, a large amout of pure human-type monoclonal antibody can be obtained.

Among the anti-OBM/sOBM monoclonal antibodies obtained by the above method, moreover, an antibody which neutralizes the biological activity of OBM/sOBM can be obtained. These antibodies which neutralize the biological activity of OBM/sOBM are expected as medicaments, particularly agents for preventing and/or treating bone metabolism abnormality, since they can inhibit the biological action (an activity to promote osteoclast formation) of OBM/sOBM in a living body. The activity of the anti-OBM/sOBM antibody to neutralize the biological activity of OBM or sOBM can be determined as an activity to inhibit osteoclast formation in an *in vitro* system for examining osteoclast formation. As *in vitro* assay systems, the following three methods can be used. That is, *in vitro* culture systems for examining osteoclastogenesis include: (1) a co-culture system of a mouse osteoblast-like stromal cell line, ST2, and mouse spleen cells in the presence of the active-form of vitamin D_3 and dexamethasone; (2) a co-culture system in which OBM is expressed on a monkey kidney cell line, COS-7, and fixed with formaldehyde, and then mouse spleen cells are cultured on the cells in the presence of M-CSF; and (3) a system of culturing mouse spleen cell in the presence of recombinant sOBM and M-CSF; however, other systems can be also used. When an anti-OBM/sOBM antibody is added to such a culture system in various concentration and its effect on osteoclastogenesis is examined, an activity of the anti-OBM/sOBM antibody to inhibit osteoclastogenesis can be measured. Also, the activity of the anti-OBM/sOBM antibody to inhibit osteoclastogenesis can be determined as an activity to suppress bone resorption *in vivo* using an experimental animal. That is, there is an animal model, an overiectomized mouse, in which osteoclastogenesis is increased. An anti-OBM/sOBM antibody is administered to such a kind of experimental animal, and an activity to inhibit bone resorption (an activity to reinforce bone mineral density) is measured. Thereby, an activity of the anti-OBM/sOBM antibody to inhibit osteoclastogenesis can be determined.

The obtained antibody, which neutralizes the biological activity of OBM/sOBM is useful as a medicament, particularly as a pharmaceutical composition for preventing and/or treating bone metabolism abnormality, or as an antibody used in immunological diagnosis of

such a disease. The antibody of the present invention can be prepared in a formulation, and administered orally or parenterally. A formulation comprising the antibody of the present invention is administered safely to human or animal as a pharmaceutical composition comprising the antibody which recognizes OBM and/or sOBM as an active ingredient. Illustrative examples of the formulation of the pharmaceutical composition include injectable solutions such as drip, suppository, nasogastric agent, sublingual agent and transdermal egent. Since the monoclonal antibody has a high molecular weight, its adsorption to glass containers such as vial and syringe tube is significant. Furthermore, the antibody is unstable and easily inactivated due to various physicochemical factors such as heat, pH, and humidity. Thus, to stably formulate the antibody, stabilizer, pH adjuster, buffer, solubilizing agent, surfactant and the like are added thereto. Illustrative examples of the stabilizer include amino acids such as glycine and alanine, saccharides such as dextran 40 and mannose, and sugar alcohols such as sorbitol, mannitol and xylitol. These may be used in combinations of two or more. These stabilizers are preferably added in an amount which is 0.01 to 100 times, particularly 0.1 to 10 times, as much as the weight of the antibody. By addition of these stabilizers, the storage stability of liquid formulation or freeze-dried formulation can be improved. Illustrative examples of buffer include phosphate buffer and citricate buffer. The buffer adjusts the pH of an aqueous formulation or a reconstituted solution of freeze-dried formulation, and thereby contributes to the stability and solubility of the antibody therein. The amount of the buffer is preferably, for example, 1 to 10 mM in an aqueous formulation or a reconsituted solution of freeze-dried formulation. The surfactant is preferably polysorbate 20, PLURONIC® (BASF Co., Co.) F-68 and polyethylene glycol, particularly preferably polysorbate 20. These may be used in combinations of two or more. A protein having high molecular weight like an antibody is liable to adsorb to glass or resin, which a container is made of. However, by addition of a surfactant adsorption of the antibody to a container in an aqueous formulation or a reconsituted solution of freeze-dried formulation can be prevented. The surfactant is preferably added in an amount of 0.001 to 1.0% of the weight of an aqueous formulation or a reconsituted solution of freeze-dried formulation. The formulation comprising the antibody of the present invention can be prepared by addition of the stabilizer, buffer and adsorption-preventing agent as described above. Particularly, when it is used as an injectable formulation for medical applications or treating animals, acceptable osmotic pressure ratio is preferably 1 or 2. The osmotic pressure ratio can be adjusted by increasing or decreasing sodium chloride in formulation. The content of the antibody in the formulation can be adjusted appropriately, dependent on the disease to be treated with said formulation, administration route and the like. The dose of the human-type antibody administered to humans depends on the affinity of the antibody to human OBM/sOBM, that

is, the dissociation constant (Kd value) of the anbibody to human OBM/sOBM. The higher the affinity is (or the lower the Kd value is), the smaller the dose that is required to exhibit medicinal benefits. Furthermore, since the half-life time of human-type antibodies in human blood is about 20 days, the human-type antibody can be administered to humans in a dose of about 0.1 to 100 mg/kg at least once within 1 to 30 days, for example.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the results of SDS-PAGE of mouse OBM protein of Example 3 of the present invention, wherein:

(A): Lane 1: molecular weight marker,

Lane 2: partially purified fraction eluted with Gly-HCl (pH 2.0), which was derived from ST2 cells cultured in the presence of the active-form of vitamin D_3 and dexamethasone,

Lane 3: partially purified fraction eluted with Gly-HCl (pH 2.0), which was derived from ST2 cells cultured in the absence of the active-form of vitamin D_3 and dexamethasone,

(B): Lane 1: molecular weight marker,

Lane 2: mouse OBM protein (Example 3) of the present invention purified with reversed phase high performance liquid chromatography.

Fig. 2 shows the results of binding experiment of the ^{125}I-labeled OCIF to an osteoblast-like stromal cell, ST2, in Example 4.

Fig. 3 shows the osteoclastogenesis-supporting abctivity of osteoblast-like stromal cell line, ST2, with different passage numbers, in Example 5(1), wherein:
1: osteoclastogenesis-supporting activity of ST2 cells with a passage number of around 10's,
2: osteoclastogenesis-supporting activity of ST2 cells with a passage number of around 40's.

Fig. 4 shows a change in expression of the protein of the present invention on an osteoblast-like stromal cell membrane, said cells were cultured in the presence of an active-form of vitamin D_3 and dexamethasone, with passage of time, in Example 5(2).

Fig. 5 shows a change in osteoclastogenesis in the co-culture system, with passage of time, of Example 5(2).

Fig. 6 shows osteoclastogenesis-inhibiting effects when OCIF was treated only during various culturing periods in the co-culture period of Example 5(3).

Fig. 7 shows the results of crosslinking experiment of the ^{125}I-labeled OCIF with the protein of the present invention, in Example 6, wherein:

Lane 1: ^{125}I-labeled OCIF-CDD1,

Lane 2: sample resulting from crosslinking of ^{125}I-labeled OCIF-CDD1 with an ST2 cell line,

Lane 3: sample resulting from crosslinking an ST2 cell in the presence of a 400-fold higher concentration of unlabeled OCIF than that of ^{125}I-OCIF.

Fig. 8 shows the results of SDS-PAGE in Example 9, wherein:

Lane 1: precipitate resulting from immuno precipitation of the protein of COS-7 cells transfected with pOBM291 without OCIF,

Lane 2: precipitate resulting from immuno precipitation of the protein of COS-7 cells transfected with pOBM291 with OCIF.

Fig. 9 shows the results of binding experiment of ^{125}I-labeled OCIF to COS-7 cells transfected with pOBM291 in Example 10, wherein:

Lanes 1 and 2: amount of ^{125}I-labeled OCIF bound to COS-7 cells transfected with pOBM291,

Lanes 3 and 4: amount of ^{125}I-labeled OCIF bound to COS-7 cells transfected with pOBM291 in the presence of a 400-fold higher concentration of unlabeled OCIF than that of ^{125}I-OCIF.

Fig. 10 shows the results of crosslinking experiment using ^{125}I-labeled OCIF in Example 11, wherein:

Lane 1: ^{125}I-labeled OCIF,

Lane 2: sample resulting from crosslinking of ^{125}I-labeled OCIF with COS-7 cells transfected with pOBM291,

Lane 3: sample resulting from crosslinking of ^{125}I-labeled OCIF with COS-7 cells transfected with pOBM291 in the presence of a 400-fold higher concentration of unlabeled OCIF than that of ^{125}I-OCIF.

Fig. 11 shows the results of Northern blot in Example 12, wherein:

Lane 1: RNA derived from ST2 cells cultured in the absence of vitamin D and dexamethasone,

Lane 2: RNA derived from ST2 cells cultured in the presence of vitamin D and dexamethasone.

Fig. 12 shows OCIF binding ability of a protein in the conditioned medium when the concentration of OCIF was varied in Example 14-(2), wherein:

○: pCEP4,

●: pCEP sOBM.

Fig. 13 shows the OCIF binding ability of the protein in the conditioned medium when the amount of the conditioned medium was varied in Example 14-(2), wherein:

○: pCEP4,

●: pCEP sOBM.

Fig. 14 shows the results of SDS-PAGE of a fusion protein of thioredoxin and mouse OBM expressed in *E. coli*, in Example 15-(2), wherein:

Lane 1: molecular weight marker,

Lane 2: soluble protein fraction derived from GI724/pTrxFus,

Lane 3: soluble protein fraction derived from GI724/pTrxOBM25.

Fig. 15 shows OCIF binding abilities when the amount of the soluble protein fractions were varied in Example 15-(3), wherein:

□: GI724/pTrxFus,

○: GI724/pTrxOBM25.

Fig. 16 shows the OCIF binding abilities of the soluble protein fractions (1%) when the concentration of OCIF was varied in Example 15-(3), wherein:

□: GI724/pTrxFus,

○: GI724/pTrxOBM25.

Fig. 17 shows the results of inhibition of the specific binding of the mouse protein obtained by expressing the mouse OBM cDNA of the present invention and purifying (mouse OBM) and the purified natural-type OCIF binding protein to OCIF, by an anti-mouse OBM rabbit antibody, wherein:

1: purified recombinant OBM treated with an antibody + ^{125}I-OCIF,

2: the purified natural-type protein treated with an antibody + ^{125}I-OCIF,

3: purified recombinant OBM untreated with an antibody + ^{125}I-OCIF,

4: the purified natural-type protein untreated with an antibody + ^{125}I-OCIF,

5: 3 + unlabeled OCIF (400-fold higher concentration than that of ^{125}I-OCIF),

6: 4 + unlabeled OCIF (400-fold higher concentration than that of ^{125}I-OCIF).

Fig. 18 shows the results of SDS-PAGE of human OBM protein expressed by the cDNA of the present invention, wherein:

Lane 1: molecular weight marker,

Lane 2: precipitate resulting from immuno precipitation of the protein derived from COS-7 cells transfected with an expression vector (phOBM) containing the cDNA of the present invention by an anti-OCIF rabbit polyclonal antibody without OCIF,

Lane 3: precipitate resulting from immuno precipitation of the protein derived from COS-7 cells transfected with an expression vector (phOBM) containing the cDNA of the present invention by an anti-OCIF rabbit polyclonal antibody with OCIF.

Fig. 19 shows the results of a binding experiment of OCIF to COS-7 cells transfected with an expression vector (phOBM) containing the cDNA of the present invention, wherein:

Lane 1: COS-7 cells transfected with phOBM + ^{125}I-OCIF,

Lane 2: COS-7 cells transfected with phOBM + ^{125}I-OCIF + a 400-fold higher concentration of unlabeled OCIF than that of ^{125}I-OCIF.

Fig. 20 shows the results of crosslinking experiment of human OBM protein encoded by the cDNA of the present invention with ^{125}I-OCIF (monomer type), wherein:

Lane 1: ^{125}I-OCIF,

Lane 2: sample resulting from crosslinking of ^{125}I-OCIF with a protein on the membrane of COS-7 cells transfected with phOBM,

Lane 3: sample resulting from crosslinking of ^{125}I-OCIF with a protein on the membrane of COS-7 cells transfected with phOBM in the presence of a 400-fold higher concentration of unlabeled OCIF than that of ^{125}I-OCIF.

Fig. 21 shows the OCIF binding ability of a protein (secretory-type human OBM) in the conditioned medium when the concentration of OCIF was varied in Example 24-(2), wherein:

○: conditioned medium of 293-EBNA cell transfected with pCEP4 vector not containing cDNA which encodes the secretory-type human OBM,

●: conditioned medium of 293-EBNA cell transfected with pCEPshOBM expression vector containing cDNA which encodes the secretory-type human OBM.

Fig. 22 shows the OCIF binding ability of the protein (secretory-type human OBM) in the conditioned medium when the amount of the conditioned medium to be added was varied while the concentration of OCIF was kept constant, in Example 24-(2), wherein:

○: conditioned medium of 293-EBNA cells transfected with pCEP4 vector not containing cDNA which encodes the secretory-type human OBM,

●: conditioned medium of 293-EBNA cells transfected with pCEPshOBM expression vector containing cDNA which encodes the secretory-type human OBM.

Fig. 23 shows the results of SDS-PAGE of a fusion protein of thioredoxin and human OBM, expressed in *E. coli*, wherein:

Lane 1: molecular weight marker,

Lane 2: soluble protein fraction derived from *E. coli* GI724/pTrxFus,

Lane 3: soluble protein fraction derived from *E. coli* GI724/pTrxOBM.

Fig. 24 shows the ability of a fusion protein to bind OCIF when the amount of soluble protein fraction containing the fused protein of thioredoxin and human OBM expressed in *E. coli* was varied, in Example 25-(3), wherein:

○: soluble protein fraction derived from *E. coli* GI724/pTrxFus,

●: soluble protein fraction derived from *E. coli* GI724/pTrxshOBM.

Fig. 25 shows the ability of the fusion protein of thioredoxin and human OBM in a soluble protein fraction of *E. coli* to bind OCIF when the concentration of OCIF was varied, in Example 25-(3), wherein:

○: soluble protein fraction derived from *E. coli* GI724/pTrxFus

●: soluble protein fraction derived from *E. coli* GI724/pTrxshOBM.

Fig. 26 shows the results of measurement of human OBM and human sOBM by

sandwich ELISA using an anti-human OBM/sOBM rabbit polyclonal antibody of the present invention, wherein:

□: human OBM,

●: human sOBM.

Fig. 27 shows the results of measurement of human OBM and human sOBM by sandwich ELISA using an anti-human OBM/sOBM monoclonal antibody of the present invention, wherein:

□: human OBM,

●: human sOBM.

Fig. 28 shows the results of measurement of mouse OBM and mouse sOBM by sandwich ELISA using an anti-human OBM/sOBM monoclonal antibody of the present invention, said antibody has cross-reactivity to both mouse OBM and mouse sOBM, wherein:

□: mouse OBM,

●: mouse sOBM.

Fig. 29 shows an activity of a fusion protein of thioredoxin and mouse OBM to promote the formation of human osteoclast-like cells

Fig. 30 shows suppression of vitamin D_3-stimulated bone resorption by an anti-OBM/sOBM antibody.

Fig. 31 shows suppression of prostaglandin E_2 (PGE_2)-stimulated bone resorption by an anti-OBM/sOBM antibody.

Fig. 32 shows suppression of parathyroid hormone (PTH)-stimulated bone resorption by an anti-OBM/sOBM antibody.

Fig. 33 shows suppression of interleukin 1 α (IL-1)-stimulated bone resorption by an anti-OBM/sOBM antibody.

BEST MODE FOR PRACTICING THE INVENTION

[Examples]

The present invention is explained in more detail with reference to the following Examples. However, these Examples are only exemplary and shall not limit the present invention thereto in any way.

[Example 1]

Production of the Protein of the Present Invention

(1) Large Scale Culture of ST2 Cells

Mouse osteoblast like stromal cell line, ST2, (Riken Cell Bank, RCB0224) was cultured with α-MEM medium containing 10% bovine fetal serum. After cultured to become confluent in a 225-cm^2 T flask for adherent cells, ST2 cells were treated with trypsin,

stripped from the T flask, washed, and then transferred to five of 225-cm^2 T flask. After addition of 60 ml of α-MEM medium containing 10^{-8} M of the active-form of vitamin D_3 (calcitriol), 10^{-7} M dexamethasone and bovine fetal serum, the resulting cells were cultured in a CO_2 incubator for 7 to 10 days. The cultured ST2 cells were recovered using a cell scraper and stored at -80°C until use.

(2) Preparation of Membrane Fraction and Solubilization of Membrane-Bound Protein

To ST2 cells (amount: about 12 ml) described in Example 1-(1) which were cultured with 80 of 225-cm^2 T flasks, a 3-fold volume (36 ml) of 10 mM Tris-hydrochloric acid buffer (pH 7.2) containing protease inhibitors (2 mM APMSFP, 2 mM EDTA, 2 mM o—phenanthroline, 1 mM leupeptin, 1 μg/ml pepstatin A and 100 units/ml aprotinin) were added. After these cells were vigorously agitated by use of a vortex mixer for 30 seconds, they were left to stand on ice for 10 minutes. Using a homogenizer (Dounce Tissue Grinder, A syringe, Wheaton Scientific Co., Ltd.), these cells were crushed. To the crushed cell solution, an equal volume (48 ml) of 10 mM Tris-hydrochloric acid buffer (pH 7.2) containing the above protease inhibitors, 0.5 M sucrose, 0.1 M potassium chloride, 10 mM magnesium chloride and 2 mM calcium chloride was added. The obtained mixture was agitated and then centrifuged at 600 x g at 4°C for 10 minutes. Through this centrifugation, cell nuclei and uncrushed cells were separated as precipitated fractions. A supernatant obtained after centrifugation was further centrifuged at 150,000 x g at 4°C for 90 minutes, and membrane fractions of the ST2 cells were obtained as precipitated fractions. To the membrane fractions, 8 ml of 10 mM Tris-hydrochloric acid buffer (pH 7.2) containing the above protease inhibitors, 150 mM of sodium chloride, and 0.1 M sucrose was added, and then 200 μl of 20% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, aigma Co., Ltd.) was added. The mixture was agitated at 4°C for 2 hours. This solution was centrifuged at 150,000 x g at 4°C for 60 minutes, and the resulting supernatant was obtained as a solubilized membrane fraction.

[Example 2]

Purification of the Protein of the Present Invention

(1) Preparation of OCIF-Immobilized Affinity Column

Isopropanol in a HITRAP® NHS-activated column (1 ml, Pharmacia Co., Ltd.) was substituted with 1 mM hydrochloric acid, and 1 ml of 0.2 M $NaHCO_3$/0.5 M NaCl (pH 8.3) solution containing 13.0 mg of recombinant OCIF prepared in accordance with a method described in WO 96/26217 was added to the column using a syringe (5 ml, Terumo Corporation). After the column was allowed to undergo a coupling reaction at room temperature for 30 minutes, 3 ml of 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) and 3 ml of 0.1 M acetic acid/0.5 M NaCl (pH 4.0) were loaded on the column alternately three times each in

total so as to inactivate excessive activated groups. Then, the mobile phase of the column was substituted again with 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) and then left to stand at room temperature for 1 hour. Thereafter, the resulting column was washed twice with 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) and 0.1 M acetic acid/0.5 M NaCl (pH 4.0) and then the mobile phase was substituted with 50 mM Tris/1M NaCl/0.1% CAHPS buffer (pH 7.5).

(2) Purification of the Protein of the Present Invention by an OCIF-Immobilized Affinity Column

Purification of OCIF binding protein was carried out at 4°C unless otherwise stated. The above OCIF-immobilized affinity column was equilibrated with 10 mM Tris-hydrochloric acid buffer (pH 7.2) containing the protease inhibitors described in Example 1-(2), 0.15 M sodium chloride and 0.5% CHAPS. To this column, about 8 ml of the solubilized membrane fraction described in Example 1-(2) was applied at a flow rate of 0.01 ml/min. The column was washed with the above 10 mM Tris-hydrochloric acid buffer (pH 7.2) containing the protease inhibitors, 0.15 M sodium chloride and 0.5% CHAPS at a flow rate of 0.5 ml/min for 100 minutes. Then, the proteins were eluted from the column with 0.1 M glycine-hydrochloric acid buffer (pH 3.3) containing the above protease inhibitors, 0.2 M sodium chloride and 0.5% CHAPS at a flow rate of 0.1 ml/min for 50 minutes. Similarly, a 0.1 M sodium citrate buffer (pH 2.0) containing said protease inhibitors, 0.2 M sodium chloride and 0.5% CHAPS was fed to the column at a flow rate of 0.1 ml/min for 50 minutes so as to elute proteins adsorbed to the column. The eluates were fractionated as 0.5 ml/fraction each. The fractions were immediately neutralized by addition of a 2M Tris solution. The fractions (the volume of the eluate was 1.0 to 5.0 ml) eluted with the buffer were concentrated to 50 to 100 µl using CENTRICON®-10 (Amersham Co., Ltd.). Aliquots of the concentrated fractions were subfractionated, and after addition of OCIF to the aliquots, they were immunoprecipitated with an anti-OCIF polyclonal antibody. After the precipitated fractions were treated with SDS, they were subjected to SDS-PAGE, and then a fraction (Fr. Nos. 3-10) showing a band of the protein having an activity to specifically bind OCIF was identified as the protein fraction of the present invention.

(3) Purification of the Protein of the Present Invention by Gel Filtration

The OCIF binding protein eluted with 0.1 M glycine-hydrochloric acid buffer (pH 3.3) and subsequently 0.1 M sodium citrate buffer (pH 2.0) after purification and concentration in accordance with the method described in Example 2-(2) was subjected to a SUPEROSE® 12 HR10/30 column (Pharmacia Co., Ltd., 1.0 X 30 cm) equilibrated with 10 mM Tris-HCl, 0.5 M NaCl and 0.5% CHAPS (pH 7.0) and developed using the above equilibration buffer as a mobile phase at a flow rate of 0.5 ml/min, and then fractions of 0.5 ml were collected. The fraction containing the protein of the present invention (Fr. Nos. 27-

32) was identified and concentrated by means of CENTRICON®-10 (Amersham Co., Ltd.) in the same manner as described above.

(4) Purification by Reversed Phase High Performance Liquid Chromatography

OCIF binding protein purified by the above gel filtration was added to a C_4 column (2.1 X 250 mm, Vydac, USA) equilibrated with 0.1% trifluoroacetic acid (TFA) and 30% acetonitrile. Elution was carried out at a flow rate of 0.2 ml/min with the gradient of acetonitrile concentration of from 30% to 55% for 50 minutes and then of from 55% to 80% for another 10 minutes. The peaks of eluted proteins were detected at 215 mm. The eluted protein of each peak was fractionated, and the peak of the protein of the present invention was identified. Thus, a highly purified protein of the present invention was obtained.

[Example 3]

SDS-PAGE of the Purified Protein of the Present Invention

First, a solubilized membrane fraction prepared from ST2 cells which were cultured in the presence or absence of the active-form of vitamin D_3 was purified with the OCIF-immobilized affinity column as described above, and the purified samples were subjected to SDS-PAGE. As shown in Fig. 1(A), it was revealed that a major protein band of about 30,000 to 40,000 was detected only in the purified sample obtained from the ST2 cells cultured in the presence of the active-form of vitamin D_3, and that a protein which specifically binds OCIF, i.e., the protein of the present invention, is selectively concentrated and purified with the OCIF-immobilized affinity column. However, in addition to the protein of the present invention, some other bands of proteins which were nonspecifically bound to the carriers, spacers or the like of the OCIF-immobilized column were also detected in both purified samples. These proteins other than the protein of the present invention were removed by gel filtration and C_4 reversed phase chromatography as described above. The SDS-PAGE of the obtained highly purified protein of the present invention is shown in Fig. 1(B). The highly purified protein of the present invention was electrophoretically homogeneous, and the molecular weight thereof was about 30,000 to 40,000.

[Example 4]

Examining the Binding of OCIF to Osteoblasts

(1) Preparation of ^{125}I-Labeled OCIF

OCIF was ^{125}I-labeled by Iodogen method. More specifically, 20 µl of 2.5 mg/ml Iodogen-chloroform solution was transferred to a 1.5 ml Eppendorf tube, and chloroform was evaporated at 40°C so as to prepare an Iodogen-coated tube. After the tube was washed three times with 400 µl of 0.5 M sodium phosphate buffer (Na-Pi, pH 7.0), 5 µl of 0.5 M Na-Pi (pH 7.0) was added thereto. Immediately after 1.3 µl (18.5 MBq) of Na-^{125}I solution (Amersham Co., Ltd., NEZ-033H20) was added to the tube, 10 µl of 1 mg/ml rOCIF

solution (monomer type or dimmer type) was added. The obtained solution was agitated with a vortex mixer, and then left to stand at room temperature for 30 seconds. The solution was transferred to a tube containing 80 μlof 10 mg/ml potassium iodide and 0.5 M Na-Pi solution (pH 7.0), and 5 μl of phosphate buffered saline solution containing 5% bovine serum albumin, and then agitated. This solution was applied to a spin column (1 ml, G-25 fine, Pharmacia Co., Ltd.) equilibrated with a phosphate buffered saline solution containing 0.25% bovine serum albumin and centrifuged at 2,000 rpm for 5 minutes. After adding 400 μl of phosphate buffered saline solution containing 0.25% bovine serum albumin to the fraction eluted from the column and subsequently mixed, 2 μl aliquots were collected, and the radioactivity thereof was measured with a gamma counter. The radiochemical purity of the thus prepared ^{125}I-labeled OCIF solution was determined by measuring the radioactivity of a fraction precipitated by 10% TCA. Furthermore, the biological activity as OCIF the ^{125}I-labeled OCIF solution was determined in accordance with a method described in WO 96/26217. Moreover, the concentration of ^{125}I-labeled OCIF was measured by ELISA in the following manner.

(2) Measurement of Concentration of ^{125}I-Labeled OCIF by ELISA

100 μl of 50 mM $NaHCO_3$ (pH 9.6) in which 2 μg/ml of anti-OCIF rabbit polyclonal antibody described in WO 96/26217 was dissolved was added to each well of 96-well immunoplate (MaxiSorp, Nunc Co., Ltd.) and left to stand at 4°C overnight. After this solution was removed, 300 μl of BLOCKACE (Snow Brand Milk Products Co., Ltd.)/phosphate buffered saline solution (25/75) was added to each well and then left to stand at room temperature for 2 hours. After this solution was removed, each well was washed three times with phosphate buffered saline solution (P-PBS) containing 0.01% polysorbate 80. Thereafter, 300 μl of BLOCKACE/phosphate buffered saline solution (25/75) containing ^{125}I-labeled OCIF sample or standard OCIF was added to each well and left to stand at room temperature for 2 hours. After this solution was removed, each well was washed six times with 200 μl of P-PBS. Then, 100 μl of BLOCKACE (Snow Brand Milk Products Co., Ltd.)/phosphate buffered saline solution (25/75) containing peroxidase-labeled anti-OCIF rabbit polyclonal antibody was added to each well and left to stand at room temperature for 2 hours. After this solution was removed, each well was washed six times with 200 μl of P-PBS. Then, 100 μl of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added to each well and then left to stand at room temperature for 2 to 3 minutes. Thereafter, 100 μl of Stopping Reagent (Scytek Co., Ltd.) was added to each well. The absorbance of each well at 490 nm was measured with a microplate reader. The concentration of the ^{125}I-labeled OCIF was calculated from a calibration curve made by using

standard OCIF.

(3) Examining the Binding of OCIF to Osteoblasts or Pancreas Cells

Mouse osteoblast-like stromal cell line, ST2, or mouse pancreas cells were suspended in α-MEM medium containing 10% bovine fetal serum (FBS) with or without 10^{-8} M of the active-form of vitamin D_3 (calcitriol) and 10^{-7} M dexamethasone at a concentration of 4×10^4 cell/ml and 2×10^6 cell/ml, respectively. 1 ml of this medium was seeded in a 24 well microplate. After the cells were cultured in a CO_2 incubator for 4 days and washed with α-MEM medium, 200 μl of medium for the binding experiment (α-MEM medium containing 0.2% bovine serum albumin, 20 mM Hepes buffer and 0.2% NaN_3) further containing 20 ng/ml of the above ^{125}I-labeled OCIF (monomer type or dimmer type) was added to each well. Furthermore, 200 μg/ml of the medium for the binding experiment containing 8 μg/ml rOCIF (400-fold higher concentration) was added to other wells which were subjected to measurements of nonspecific binding. After the cells were cultured in a CO_2 incubator for 1 hour, they were washed three times with 1 ml of phosphate buffered saline solution. Since pancreas cells are floating cells, cells in each well were washed in the 24-well plate with centrifugation. After washing, 500 μl of 0.1 N NaOH solution was added to each well and left to stand at room temperature for 10 minutes. Thereby, the cells were washed, and the amount of RI bound to the cells was measured with a gamma counter.

The ^{125}I-labeled OCIF did not bind the cultured pancreas cells, but specifically bound only osteoblast like stromal cells cultured in the presence of the active-form of vitamin D_3. Thereby, it was revealed that the protein of the present invention was a membrane-bound protein induced on the cell surface of osteoblast like stromal cells with the active-form of vitamin D_3 and dexamethasone.

[Example 5]

Biological Activity of the Protein of the Present Invention

(1) Ability of Osteoblast Like Stromal Cells to Support Osteoclast Formation

The ability of osteoblasts to support osteoclast formation was examined by measuring tartaric acid resistant acid phosphatase activity (TRAP activity) of the formed osteoclasts. More specifically,mouse osteoblast-like stromal cell line, ST2, (5×10^3 cells/100 μl/well) (2×10^5 cells/100 μl/well) and pancreas cells derived from a ddy mouse (8 to 12 weeks old) were suspended in α-MEM medium containing 10% bovine fetal serum, 10^{-8} M of the active-form of vitamin D_3 and 10^{-7} M dexamethasone and seeded in a 96-well plate. After the cells were cultured in a CO_2 incubator for one week, each well was washed with phosphate buffered saline solution. Then, 100 μl of ethanol/acetone (1:1) was further added to the wells and fixed at room temperature for 1 minute. Then, 100 μl of 50 mM citric

acid buffer (pH 4.5) containing 5.5 mM p-nitrophenol phosphate and 10 mM sodium tartrate was added to each well and then allowed to react at room temperature for 15 minutes. After the reaction, 0.1 N NaOH solution was added to each well, and the absorbance at 405 nm was measured with a microplate reader. Fig. 3 shows the results of examining the abilities of ST2 cells to support osteoclast formation, wherein the passage number of said cells were around 10's or around 40's (after purchased from Riken Cell Bank). From these results, it was revealed that ST2 cells of a high passage number had a high ability to support osteoclast formation.

(2) Changes with the Passage of Time in Expression of the Protein of the Present Invention on the Menbrane of Osteoblast Like Stromal Cells Cultured in the Presence of the Active-Form of Vitamin D_3 and Dexamethasone and Those in Osteoclast Formation in a Co-Culture System

Osteoblast-like stromal cell line, ST2, was cultured in the presence of the active-form of vitamin D_3 and dexamethasone for 7 days in the same manner as in Example 4-(3). OCIF-binding experiment was conducted using ^{125}I-labeled OCIF (monomer type) described in Example 4-(1). Nonspecific binding was measured by competing the ^{125}I-labeled OCIF with a 400-fold higher concentration of unlabeled OCIF in binding to ST2 cells. As a result, the amount of specific binding of the ^{125}I-labeled OCIF was increased, due to the active-form of vitamin D_3 and dexamethasone, with an increase in culturing days. That is, as shown in Figures 4 and 5, the protein of the present invention was expressed on the cell surface of ST2 cells due to the active-form of vitamin D_3 with an increase in culturing days, and its expression reached maximum on the fourth day of culture. On the other hand, osteoclast-like cells were formed after co-culture of mouse spleen cells and ST2 cells in the presence of the active-form of vitamin D_3. TRAP (a marker enzyme for osteoclasts)-positive mononuclear osteoclast-like cells were formed on the third or fourth day of culture, and further, differentiated and matured TRAP-positive multinuclear cells were formed on the fifth or sixth day of culture. It was found that change with the passage of time in expression of the protein of the present invention and in osteoclast formation corresponded well with each other.

(3) Effect of Inhibiting Osteoclast Formation When OCIF Was Treated Only During a Restricted Period of Co-Culture

To further clarify that the protein of the present invention was a factor involved in osteoclast formation, cells cultured during various periods (two days each, except for the fifth day) were treated with 100 ng/ml of OCIF in the above 6-day co-culture described in Example 5-(2). As a result shown in Fig. 6, in the case where OCIF was added during the 48th to 96th hr (as counted from the beginning of culture), when the protein of the present

invention was expressed at highest level on ST2 cells, osteoclast formation was inhibited most effectively. That is, it was revealed that OCIF inhibited osteoclast formation by binding to ST2 cells via the protein of the present invention.

From the above results, it became clear that the protein of the present invention was induced on the membranes of osteoblast-like stromal cells with the active-form of vitamin D_3 and dexamethasone, and had the biological activity (effect) of a factor which supports and promotes differentiation and maturation of osteoclasts.

[Example 6]

Crosslinking Experiment of ^{125}I-labeled OCIF to the Protein of the Present Invention

To further identify the presence of the protein of the present invention, ^{125}I-labeled OCIF was allowed to crosslink with the protein of the present invention. As in Example 4-(3), mouse osteoblast like cell line, ST2, was cultured in the presence or absence of the active-form of vitamin D_3 and dexamethasone for 4 days. After the cells were washed with 1 ml of phosphate buffered saline solution, 200 µl of medium for binding experiment (α-MEM medium containing 0.2% bovine serum albumin, 20 mM Hepes buffer, 0.2% NaN_3 and 100 µg/ml heparin) futher containing 25 ng/ml of the above ^{125}I-labeled OCIF (monomer type) or 40 ng/ml of ^{125}I-labeled OCIF-CDD1 was added. ^{125}I-labeled OCIF-CDD1 was obtained by expressing the protein described as SEQ ID NO: 76 in WO 96/26217 with animal cells and labeling in accordance with the above method. Furthermore, the medium for the binding experiment, containing a 400-fold higher concentration of OCIF, was added to the other well and was subjected to an experiment for nonspecific binding. After the cells were cultured in a CO_2 incubator for 1 hour, they were washed three times with 1 ml of phosphate buffered saline solution containing 100 µg/ml of heparin. Then, 500 µl of phosphate buffered saline solution in which 100 µg/ml of crosslinking agent DSS (Disuccinimidyl suberate, Pierce Co., Ltd.) was dissolved was added thereto, and allowed to react at 0°C for 10 minutes. After the cells in these wells were washed twice with 1 ml of phosphate buffered saline solution cooled to 0°C, 100 µl of 20 mM Hepes buffer containing 1% Triton X-100, 2 mM PMSF (phenylmethylsulfonyl fluoride), 10 µM pepstatin, 10 µM leupeptin, 10 µM antipain and 2 mM EDTA was added to each well, and left to stand at room temperature for 30 minute so as to lyse the cells. After 15 µl of these samples were treated with SDS under non-reducing conditions in accordance with a commonly used method, they were run on a SDS-polyacrylamide electrophoresis gel (with a gradient of 4 to 20% polyacrylamide, Daiichi Pure Chemicals Co., Ltd.). After electrophoresis, the gel was dried and exposed to BIOMAX® MS film (Kodak Co., Ltd.) using BIOMAX® MS amplifying screen (Kodak Co., Ltd.) at -80°C for 24 hours. The exposed films were developed in accordance with a

commonly used method. When the ^{125}I-labeled OCIF (monomer type, 60 kDa) was used, a crosslinked protein having a molecular weight of about 90,000 to 110,000 was detected. On the other hand, when the ^{125}I-labeled OCIF-CDD1 (31 kDa) was used, a crosslinked protein of about 70 to 80 kDa (78 kDa on average) was detected as shown in Fig. 7.

[Example 7]

Scatchard Plot Analysis of the Protein of the Present Invention Expressed on ST2 Cells

Medium for the binding experiment (α-MEM medium containing 0.2% bovine serum albumin, 20 mM Hepes buffer and 0.2% NaN_3) further containing 1,000 pM of the above ^{125}I-labeled OCIF (monomer type) was prepared and diluted stepwise at a dilution rate of 1/2 with the medium for the binding experiment. Furthermore, another medium for determining nonspecific binding was prepared by adding a 400-fold higher concentration of unlabeled monomer type OCIF to the above medium. 200 μl of these prepared solutions were added to wells of the above ST2 cells (about 10th passage), cultured for 4 days in the presence of 10^{-8} M of the active-form of vitamin D_3 (calcitriol) and 10^{-7} M dexamethasone, and binding of the ^{125}I-labeled OCIF was tested in the same manner as in Example 4-(3). The obtained results were Scatchard-plotted in accordance with a common method, and dissociation constants of OCIF and the OCIF binding protein, and the number of the OCIF binding protein (site) per one ST2 cell were determined. As a result, the dissociation constants of OCIF and the protein of the present invention were 280 pM, and the number of the OCIF binding protein (site) per one ST2 cell was about 33,000/cell. Furthermore, a cultured ST2 cell with a passage number of around 40's had higher ability to support osteoclast formation than that with a passage number of around 10's as shown in Example 5-(1), so that the number of sites of the protein of the present invention expressed on the ST2 cell with a passage number of around 40's was measured. As a result, the number of site was 58,000/cell which, was clearly greater than that on the ST2 cell with a passage number of around 10's. It was revealed that the amount of the expression of the protein of the present invention associated with the degree of the ability of ST2 cell to support osteoclast formation. This finding indicates that the protein of the present invention is a factor to support and promote differentiation and maturation of osteoclasts.

[Example 8]

Cloning of OBM cDNA

(1) Extraction of RNA from Mouse ST2 Cell

Mouse osteoblast-like stromal cell line, ST2, (Riken Cell Bank, RCB0224) was with α-MEM medium (Gibco BRL Co., Ltd.) containing 10% bovine fetal serum. After cultured cells become confluent in 225-cm^2 T flasks for adherent cell culture, ST2 cells were treated with trypsin, stripped from the T flask, washed, and transferred to five 225-cm^2 T

flasks. After adding 60 ml of α-MEM medium containing 10^{-8} M of the active-form of vitamin D_3 (calcitriol, Wako Pure Chemical Industries, Ltd.), 10^{-7} M dexamethasone and 10% bovine fetal serum thereto, the cells were cultured in a CO_2 incubator for 5 days. Total RNA was extracted from the cultured ST2 cells using ISOGEN (Wako Pure Chemical Industries, Ltd.). Poly A^+ RNA was prepared from about 600 μg of the total RNA using an Oligo(dT)-cellulose column (5'-3' Prime Co., Ltd.). About 8 μg of poly A^+ RNA was obtained.

(2) Construction of Expression Library

Double strand cDNAs were synthesized from 2 μg of the poly A^+ RNA obtained in Example 8-(1) with Great Lengths cDNA Synthesis kit (Clontech Co., Ltd.) in accordance with a manual thereof. More specifically, 2 μg of the poly A^+ RNA and an Oligo$(dT)_{25}$(dN) primer were mixed together, distilled water was added thereto so that the final volume was 6.25 μl, and the mixture was incubated at 70°C for 3 minutes, and then cooled in ice for 2 minutes. Then, 2.2 μl of distilled water, 2.5 μl of 5X First-strand buffer, 0.25 μl of 100 mM DTT (dithiothreitol), 0.5 μl of PRIME RNase Inhibitor (1 U/ml) (5'-3' Prime Co., Ltd.), 0.5 μl of [α-^{32}P]dCTP (Amersham Co., Ltd., 3,000 Ci/mmol, 2 μCi/μl) which was diluted to be one fifth concentration, 0.65 μl of dNTP (20 mM each) and 1.25 μl (250 units) of MMLV ($RNaseH^-$) reverse transcriptase were added thereto, respectively. Thus obtained solution was incubated at 42°C for 90 minutes. Then, 62.25 μl of distilled water, 20 μl of 5X second-strand buffer, 0.75 μl of dNTP (20 mM each) and 5 μl of Second-strand enzyme cocktail were added thereto, respectively. Thus obtained solution was incubated at 16°C for 2 hours. 7.5 units of T4 DNA polymerase was added thereto, and further incubated at 16°C for another 30 minutes. Thereafter, 5 μl of 0.2 M EDTA was added to terminate the reaction, and after a phenol-chloroform treatment, ethanol precipitation was carried out. An EcoRI-SalI-NotI linker (Clontech Co., Ltd.) was added to an end of the double strand cDNA and then phosphorylated at its end. Using a column for size fractionation, cDNAs of not smaller than 500 bp were separated, and ethanol-precipitated. The precipitated DNAs were reconstituted in water and inserted into pcDL-SR α296 (Molecular and Cellular Biology, Vol. 8, pp. 466 to 472, 1988) (Takara Shuzo Co., Ltd.) previously cleaved with a resriction enzyme, EcoRI, and subsequently treated with CIAP (calf intestine alkaline phosphatase, Takara Shuzo Co., Ltd.).

(3) Screening of Expression Library in Which the Binding to OCIF Was Used as an Index

E. coli XL2 Blue MRF' (Toyobo Co., Ltd.) was transformed with the DNA obtained in Example 8-(2), and allowed to grow on a L Carbenicillin Agar Medium (1% trypton, 0.5% yeast extract, 1% NaCl, 60 μg/ml carbenicillin and 1.5% agar) prepared in a 24-well plastic plate for cell culture so that the cells was grown to about 100 colonies per

well. The transformants in each well were suspended in 3 ml of Terrific Broth ampicillin medium (1.2% trypton, 2.4% yeast extract, 0.4% glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4, 100 μg/ml ampicillin), and cultured with shaking at 37°C overnight. The *E. coli* was collected by centrifugation, and plasmid DNAs were prepared therefrom with QIAWELL® kit (QIAGEN Co., Ltd.). The DNA content was determined by detecting absorbance at 260 nm, and the DNAs was concentrated by ethanol precipitation and dissolved in distilled water so that the concentration was 200 ng/μl. Thus, 500 DNA pools each derived from about 100 colonies were prepared and used for transfection of COS-7 cells (Riken Cell Bank, RCB0539). COS-7 cells were seeded in a 24-well plate so as to achieve 8 X 10^4 cells/well and cultured in a CO_2 incubator at 37°C overnight by use of DMEM medium containing 10% bovine fetal serum. On the following day, the medium was removed, and the cells were then washed with serum-free DMEM medium. In accordance with a protocol attached to lipofectamine (Gibco Co., Ltd.) which was a reagent for transfection, the plasmid DNA previously diluted with OPTI-MEM® medium (Gibco BRL Co., Ltd.) and lipofectamine were mixed together, and after 15-minute inculation, the mixture was added to the cells in each well. The amounts of DNA and lipofectamine used were 1 μg and 4 μl, respectively. After 5-hour incubation, the medium was removed, and 1 ml of DMEM medium (Gibco BRL Co., Ltd.) containing 10% bovine fetal serum was added and cultured in a CO_2 incubator (5% CO_2) at 37°C for 2 to 3 days. The COS-7 cells obtained after transfection and subsequent culture for 2 to 3 days were washed with serum-free DMEM medium. Then, 200 μl of medium for binding experiment (serum-free DMEM medium containing 0.2% bovine serum albumin, 20 mM Hepes buffer, 0.1 mg/ml heparin and 0.02% NaN_3) further containing 20 ng/ml of ^{125}I-labeled OCIF was added thereto. Cells were cultured in a CO_2 incubator (5% CO_2) at 37°C for 1 hour and washed twice with 500 μl of phosphate buffered saline solution containing 0.1 mg/ml heparin. After washing, 500 μl of 0.1 N NaOH solution was added thereto, and then left to stand at room temperature for 10 minutes so as to lise the cells. The amount of ^{125}I in each well was measured with a gamma counter (Packard Co., Ltd.). After screening the 500 pools in total, a DNA pool containing a cDNA encoding a protein that could specifically bind OCIF was isolated. Furthermore, the DNA pools containing the cDNA of the present invention were subfractionated, and then employed to repeat the above transfection and screening. Thereafter, a cDNA encoding a protein which could bind OCIF was isolated. A plasmid containing the cDNA was referenced pOBM291. *E. coli* containing the plasmid was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology as pOBM291 with the deposition number of FERM BP-5953 on May 23, 1997. Methods for

^{125}I-labeling of OCIF and determining (the concentration of) ^{125}I-labeled OCIF by ELISA are as follows. OCIF was ^{125}I-labeled in accordance with Iodogen method. 20 µl of 2.5 mg/ml Iodogen-chloroform solution was transferred to a 1.5 ml Eppendorf tube, and chloroform was evaporated at 40°C so as to prepare an Iodogen-coated tube. After the tube was washed three times with 400 µl of 0.5 M sodium phosphate buffer (Na-Pi, pH 7.0), 0.5 µl of 0.5 M Na-Pi with a pH of 7.0 was added thereto. Immediately after 1.3 µl (18.5 MBq) of Na-^{125}I solution (Amersham Co., Ltd., NEZ-033H20) was added thereto, 10 µl of 1 mg/ml OCIF solution (monomer type or dimmer type) was added. The resulting solution was agitated with a vortex mixer and then left to stand at room temperature for 30 seconds. This solution was transferred to a tube in which 10 mg/ml potassium iodide, 80 µl of 0.5 M Na-Pi solution (pH 7.0) and 5 µl of phosphate buffered saline solution containing 5% bovine serum albumin (BSA-PBS) was previously added and then agitated. This solution was applied to a spin column (1 ml, G-25 fine, Pharmacia Co., Ltd.) equilibrated with BSA-PBS and centrifuged at 2,000 rpm for 5 minutes. After 400 µl of BSA-PBS was added to an eluate from the column and mixed, 2 µl was subfractionated and its radioactivity was measured with a gamma counter. The radiochemical purity of thus prepared ^{125}I-labeled OCIF solution was determined by measuring the radioactivity of a fraction precipitated with 10% TCA. Furthermore, the biological activity as OCIF of the ^{125}I-labeled OCIF solution was determined in accordance with a method described in WO 96/26217. Moreover, the concentration of ^{125}I-labeled OCIF was measured by ELISA in the following manner. That is, 100 µl of 50 mM $NaHCO_3$ (pH 9.6) in which 2 µg/ml of anti-OCIF rabbit polyclonal antibody described in WO 96/26217 was dissolved was added to each well of a 96-well immunoplate (Nunc Co., Ltd., MaxiSorp) and left to stand at 4°C overnight. After this solution was removed, 200 µl of a combined solution of of BLOCKACE (Snow Brand Milk Products Co., Ltd.) and phosphate buffered saline solution (mixing ratio = 25:75: B-BPB) was added to each well and then left to stand at room temperature for 2 hours. After this solution was removed, each well was washed three times with phosphate buffered saline solution (P-PBS) containing 0.01% Polysorbate 80. Thereafter, 100 µl of B-PBS containing a ^{125}I-labeled OCIF or standard OCIF was added thereto and left to stand at room temperature for 2 hours. After this solution was removed, each well was washed six times with 200 µl of P-PBS. Then, a peroxidase-labeled anti-OCIF rabbit polyclonal antibody was diluted with B-PBS and 100 µl of the diluted solution was added to each well, and then left to stand at room temperature for 2 hours. After this solution was removed, each well was washed six times with 200 µl of P-PBS. Then, 100 µl of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added to each well and then left to stand at room

temperature for 2 to 3 minutes. Thereafter, 100 µl of Stopping Reagent (Scytek Co., Ltd.) was added thereto. The absorbance of each well at 450 nm was measured with a microplate reader. The concentration of the ^{125}I-labeled OCIF was determined from a calibration curve made using standard OCIF.

(4) Determination of the Nucleotide Sequence of cDNA Which Eccodes the Full Length Amino Acid Sequence of OBM

The nucleotide sequence of OBM cDNA obtained in Example 8-(3) was determined with Taq Dye Deoxy Terminator Cycle Sequencing kit (Perkin Elmer Co., Ltd.). That is, using pOBM291 as a template, the nucleotide sequence of the inserted fragment was directly determined. Furthermore, about 1.0 kb and about 0.7 kb fragments obtained by cleaving pOBM291 with a restriction enzyme, EcoRI, were inserted into EcoRI site of plasmid pUC19 (Takara Shuzo Co:, Ltd.) and sequenced, respectively. A primer SRR2 for sequencing the DNA fragment inserted in pcDL-SR α296, primers M13PrimerM3 and M13PrimerRV (Takara Shuzo Co., Ltd.) for sequencing the DNA fragment inserted in the plasmid pUC19, and a synthetic primer OBM #8 designed based on the nucleotide sequence of OBM cDNA were used. The sequences of these primers are shown as SEQ ID NOs: 3 to 6.

Furthermore, the determined nucleotide sequence of OBM cDNA is shown as SEQ ID NO: 2, and the deduced an amino acid sequence is shown as SEQ ID NO: 1.

[Example 9]

Expression of the Protein Encoded by the cDNA of the Present Invention

COS-7 cells were transfected with plasmid pOBM291 with lipofectamine in each well of a 6-well plate, and were cultured in DMEM medium containing 10% bovine fetal serum for 2 days. The medium was replaced with cysteine/methionine-free DMEM (DAINIPPON PHARMACEUTICAL CO., LTD.) in which 5% dialyzed bovine fetal serum (800 µl/well) was added, and the cells were cultured for another 15 minutes. Then, 14 µl of Express Protein Labeling Mix (NEN CO., LTD., 10 mCi/ml) was added thereto. After the cells were cultured for 4 hours, 200 µl of DMEM medium containing 10% bovine fetal serum was added, and the cells were cultured for 1 hour. After the cells were washed twice with PBS, 0.5 ml of TSA buffer (10 mM Tris-HCl (pH 8.0) containing 0.14 M NaCl and 0.025% NaN_3) containing 1% Triton X-100, 1% bovine hemoglobin, 10 µg/ml leupeptin, 0.2 TIU/ml aprotinin and 1 mM PMSF was added, and the cells were left to stand on ice for 1 hour. After the cells were crushed by pipetting, centrifugation was carried out at 3,000 xg at 4°C and for 10 minutes so as to obtain a supernatant. To 100 µl of this supernatant, 200 µl of dilution buffer (TSA buffer containing 0.1% Triton X-100, 0.1% bovine hemoglobin, 10 µg/ml leupeptin, 0.2 TIU/ml aprotinin and 1 mM PMSF) was added, and the resulting

supernatant was shaken together with Protein A Sepharose® (50 μl) at 4°C for 1 hour, and then centrifuged at 4°C and 1,500 X g for 1 minute so as to collect a supernatant. Thereby, a fraction nonspecifically binding to the Protein A Sepharose® was removed. OCIF (1 μg) was added to this supernatant, and the obtained supernatant was shaken at 4°C for 1 hour so that OBM bound OCIF. Then, an anti-OCIF polyclonal antibody (50 μg) was added, and the solution was shaken at 4°C for 1 hour. Then, Protein A Sepharose® (10 μl) was further added, and the solution was further shaken at 4°C for another 1 hour. The solution was centrifuged at 1,500 xg at 4°C for 1 minute and the precipitated fraction was collected. The precipitate resulting from centrifugation at 1,500 xg at 4°C for 1 was washed twice with the dilution buffer, twice with the dilution buffer without bovine hemoglobin, once with TSA buffer, and once with 50 mM Tris-HCl (pH 6.5). After washing, SDS buffer (0.125 M Tris-HCl, 4% dodecyl sodium sulfate, 20% glycerol, 0.002% bromophenol blue, pH 6.8) containing 10% β mercaptoethanol was added to the precipitate. The precipitate was heated at 100°C for 5 minutes and subjected to SDS-PAGE (12.5% polyacrylamide gel, Daiichi Pure Chemicals Co., Ltd.). After the gel was fixed in accordance with a commonly used method, signals of isotope were amplified with Amplify (Amersham Co., Ltd.), and the fixed gel was exposed to BioMax® MR film (Kodak Co., Ltd.) at -80°C. The results are shown in Fig. 8. The molecular weight of the protein encoded by the cDNA of the present invention was found to be about 40,000.

[Example 10]

Binding of the Protein Encoded by the cDNA of the Present Invention to OCIF

COS-7 cells were transfected with plasmid pOBM291 with lipofectamine in wells of a 24-well plate and cultured for 2 to 3 days. Then, the cells were washed with serum-free DMEM medium, and 200 μl of medium for binding experiment (serum-free DMEM medium containing 0.2% bovine serum albumin, 20 mM Hepes buffer, 0.1 mg/ml heparin and 0.2% NaN_3) containing 20 ng/ml of ^{125}I-labeled OCIF was added thereto. Furthermore, 200 μl of the medium for binding experiment containing 8 μg/ml of unlabeled OCIF in addition to 20 ng/ml of the ^{125}I-labeled OCIF was added to other wells. The cells were cultured in a CO_2 incubator (5% CO_2) at 37°C for 1 hour, and washed twice with 500 μl of phosphate buffered saline solution containing 0.1 mg/ml heparin. After washing, 500 μl of 0.1 N NaOH solution was added to each well, and then the well was left to stand at room temperature for 10 minutes so as to lyse the cell. The amount of ^{125}I in each well was measured with a gamma counter. As a result, it was confirmed that the ^{125}I-labeled OCIF bound only to cells transfected with the plasmid pOBM291 as shown in Fig. 9. Further, it was also confirmed that the binding was significantly inhibited by addition of a 400-fold

higher concentration of (unlabeled) OCIF. From these results, it was revealed that OBM, a protein encoded by the cDNA of the plasmid pOBM291 specifically bound OCIF on the surface of COS-7 cell.

[Example 11]

Crosslinking Experiment of ^{125}I-labeled OCIF to the Protein Encoded by the cDNA of the Present Invention

In order to analyze the characteristics of the protein encoded by the cDNA of the present invention more specifically, ^{125}I-labeled monomer type OCIF was allowed to crosslink with the protein encoded by the cDNA of the present invention. COS-7 cells were transfected with plasmid pOBM291 in accordance with the method described in Example 8-(3), 200 μl of medium for binding experiment containing the above ^{125}I-labeled OCIF (25 ng/ml) was added thereto. Furthermore, the medium for the binding experiment containing a 400-fold higher concentration of unlabeled OCIF in addition to the ^{125}I-labeled OCIF was added to other wells. The cells were cultured in a CO_2 incubator (5% CO_2) at 37°C for 1 hour and washed twice with 500 μl of phosphate buffered saline solution containing 0.1 mg/ml of heparin. 500 μl of phosphate buffered saline solution containing 100 μg/ml of crosslinking agent DSS (Disuccinimidyl suberate, Pierce Co., Ltd.) was added to these cells, and the cells were allowed to react at 0°C for 10 minutes. After the reaction, the cells in these wells were washed twice with 1 ml of phosphate buffered saline solution cooled to 0°C. Then, 100 μl of 20 mM Hepes buffer containing 1% Triton X-100 (Wako Pure Chemical Industries, Ltd.), 2 mM PMSF (phenylmethylsulfonyl fluoride, Sigma Co., Ltd.), 10 μM pepstatin (Wako Pure Chemical Industries, Ltd.), 10 μM leupeptin (Wako Pure Chemical Industries, Ltd.), 10 μM antipain (Wako Pure Chemical Industries, Ltd.) and 2 mM EDTA (Wako Pure Chemical Industries, Ltd.) was added to these cells, and the wells were left to stand at room temperature for 30 minute so as to lise the cells. After 15 μl of these samples were treated with SDS under non-reducing conditions in accordance with a commonly used method, they were subjected to electrophoresis with gel for SDS-electrophoresis (gradient of 4 to 20% polyacrylamide, DAIICHI PURE CHEMICALS CO., LTD.). After electrophoresis, the gel was dried and exposed to BioMax® MS film (Kodak Co., Ltd.) with BioMax® MS amplifying screen (Kodak Co., Ltd.) at -80°C for 24 hours. The exposed films were developed in accordance with a commonly used method. As results of crosslinking of the ^{125}I-labeled monomer type OCIF with the protein encoded by the cDNA of the present invention, a band having a molecular weight of about 90,000 to 110,000 was detected as shown in Fig. 10.

[Example 12]

Northern Blot Analysis

ST2 cells were cultured to become confluent in a 25-cm^2 T flask, for culturing adherent cells, and treated with trypsin. After being stripped from the T flask, the cells were washed and seeded in a 225-cm^2 T flask. 60 ml of α-MEM medium containing 10^{-8} M of the active-form of vitamin D_3 10^{-7} M dexamethasone and 10% bovine fetal serum was added thereto, and the cells were cultured in a CO_2 incubator for 4 days. Then, total RNA was extracted from the above-cultured ST2 cells with ISOGEN (Wako Pure Chemical Industries, Ltd.). In addition, total RNA was extracted from ST2 cells cultured in the absence of the active-form of vitamin D_3 and dexamethasone in accordance with the above method. To 20 μg (4.5 μl) of each total RNA sample, 2.0 μl of 5X gel electrophoresis buffer (0.2 M morpholinopropanesulfonic acid (pH 7.0), 50 mM sodium acetate, 5 mM EDTA), 3.5 μl of formaldehyde and 10.0 μl of formamide were added. The total RNA samples were incubated at 55°C for 15 minutes and subjected to electrophoresis. Gels for electrophoresis consisted of 1.0% agarose, 2.2 M ionized formaldehyde, 40 mM morpholinopropanesulfonic acid (pH 7.0), 10 mM sodium acetate and 1 mM EDTA. Moreover, the electrophoresis was performed in buffer comprising 40 mM morpholinopropanesulfonic acid (pH 7.0), 10 mM sodium acetate and 1 mM EDTA. After the electrophoresis, the RNA was transferred to nylon membranes. About 1.0 kb DFA fragments were obtained by cleaving pOBM291 with a restriction enzyme, EcoRI, and labeled with α-^{32}P-dCTP (Amersham Co., Ltd.) using MEGAPRIME DNA Labeling Kit (Amersham Co., Ltd.), and thus used as probes for hybridization. As a result, it was revealed that gene expression of the protein (OBM) encoded by the cDNA of the present invention was strongly induced in the ST2 cells cultured in the presence of the active-form of vitamin D_3 and dexamethasone.

[Example 13]

Ability of the Protein Encoded by the cDNA of the Present Invention to Support Osteoclast Formation

In accordance with the method described in Example 8-(3), COS-7 cells were transfected with pOBM219. After 3-day incubation, the cells were treated with trypsin and then centrifuged-washed once with phosphate buffered saline solution. Then, the cells were fixed at room temperature for 5 minutes in suspension of PBS containing 1% paraformaldehyde, and then centrifuged-washed six times with PBS. Mouse spleen cells and ST2 cells were prepared with α-MEM medium containing 10^{-8}M of the active-form of vitamin D_3, 10^{-7}M dexamethasone and 10% bovine fetal serum so that the cell concentration become 1 X 10^6 cells/ml or 4 X 10^4 cells/ml and then added to a 24-well plate in a volume of 700 μl and 350 μl, respectively. Furthermore, TC insert (Nunc Co., Ltd.) was set in each

well. The fixed COS cells (350 μl) diluted stepwise with the above medium and OCIF (50 μl), were added to TC inserts and cultured at 37°C for 6 days. As a result, it was revealed that an activity of OCIF to inhibit osteoclast formation was suppressed by the protein encoded by the cDNA of the present invention.

[Example 14]

Expression of Secretory-type OBM

(1) Construction of Plasmid for Expressing Secretory-Type OBM Expression

A PCR reaction was carried out using OBM HF (SEQ ID NO: 7)/OBM XR (SEQ ID NO: 8) and pOBM291 as primers and a template, respectively. After the reaction product was purified through agarose gel electrophoresis, it was digested with the restriction enzymes, HindIII and EcoRI, and then purified through agarose gel electrophoresis again. The purified fragment (0.6 kb), HindIII/EcoRV fragment (5.2 kb) of pSec TagA (Invitrogen Co., Ltd.) and EcoRI/PmaCI fragment (0.32 kb) of OBM cDNA was subjected to ligation using Ligation Kit Ver. 2 (Takara Shuzo Co., Ltd.), and subsequently *E. coli* DH5α was transformed by the ligation product. Plasmid was purified from the obtained ampicillin-resistant strains by alkaline-SDS method and then cleaved with restriction enzymes so as to select a plasmid wherein 0.6 kb and 0.32 kb of fragments were inserted into pSec TagA. The selected plasmid was subjected to sequencing with Dye Terminator Cycle Sequencing FS kit (Perkin Elmer Co., Ltd.), thereby it was confirmed that the plasmid had the sequence encoding secretory-type OBM (nucleotides 338-1355 of SEQ ID NO: 2, amino acids 72-316 of SEQ ID NO: 1). After the plasmid was digested with restriction enzymes, Nhel and Xhol, a fragment (1.0 kb) corresponding to secretory-type OBM cDNA was collected by agarose gel electrophoresis. This fragment was inserted into a NheI/XhoI digested expression vector, pCEP4 (10.4 kb) (Invitrogen Co., Ltd.), by using the ligation kit, and *E. coli* DH5α were transformed with the ligation product. Plasmid was purified, from the ampicillin-resistant strains obtained, by alkaline-SDS method, and digested with the restriction enzymes. Then the plasmid was analyzed so as to select *E. coli* strains which had a plasmid for expressing secretory-type OBM (pCEP sOBM) with the desired structure. An *E. coli* strain having the pCEP sOBM was cultured, and the pCEP sOBM was purified therefrom with QIA® Filter Plasmid Midi Kit (QIAGEN CO., LTD.).

(2) Expression of Secretory-Type OBM

293-EBNA cells were suspended in IMDM containing 10% FCS (IMDM-10% FCS), and seeded in a collagen-coated 24 well plate (SUMITOMO BAKELITE CO., LTD.) so that the cell concentration was 2 X 10^5 cells/2 ml/well, and cultured overnight. The cells were transfected with 1 μg of pCEP sOBM or pCEP4 using 4 μl of lipofectamine

(GIBCO CO., LTD.), and then cultured in 0.5 ml of serum-free IMDM or IMDM-10% FCS for another 2 days. Thereafter, the conditioned medium was collected. Expression of secretory-type OBM in the conditioned medium was confirmed in the following manner. After sodium hydrogencarbonate was added to the conditioned medium so that the final concentration was 0.1 M, the culture solution was added to a 96-well plate, and left to stand at 4°C overnight. Then the OBM in the conditioned medium was immobilized in the 96-well plate. This plate was left to stand for blocking at room temperature for 2 hours by use of BLOCKACE (Snow Brand Milk Products Co., Ltd.) solution diluted with PBS to be one forth concentration (B-PBS). Then, 100 µl of 3-100 ng/ml OCIF diluted with B-PBS was added to each well, and the wells were left to stand at 37°C for 2 hours. After washing the plate with PBS containing 0.05% Tween 20 (PBS-T), 100 µl of peroxidase-labeled anti-OCIF rabbit polyclonal antibody, which was described in WO 96/26217, diluted with B-PBS was added to each well, and the cells were left to stand at 37°C for 2 hours. After washing each well with PBS-T six times, 100 µl of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added thereto and then left to stand at room temperature for about 10 minutes. Thereafter, 100 µl of Stopping Reagent (Scytek Co., Ltd.) was added to each well. The absorbance of each well at 450 nm was measured with a microplate reader. The results are shown in Fig. 12. In the plate in which substances included in the conditioned medium of the cells transduced by pCEP sOBM were immobilized, absorption at 450 nm increased in the OCIF concentration-dependent manner. On the other hand, in the plate in which substances included in the conditioned medium of the cells transduced by pCEP4 vector were immobilized, no increase in absorption at 450 nm was observed. Furthermore, Fig. 13 shows the results of experiments when the amount of the conditioned medium applied to the immobilization was varied within a range of 5 to 90% and a constant concentration of OCIF (50 ng/ml) was futher added. In the plate in which substances included in the conditioned medium of the cells transduced by pCEP sOBM were immobilized, absorption at 450 nm increased corresponding to an increase in the amount of the conditioned medium. On the other hand, in the plate in which substances included in the conditioned medium of the cells transduced by pCEP4 vector were immobilized, no increase in absorption was observed. From these results, secretory-type OBM was confirmed to be produced in the conditioned medium of the cells transduced by pCEP sOBM.

[Example 15]

Expression of Thioredoxin-OBM Fusion Protein (Trx-OBM)

(1) Construction of a Vector for Expressing Thioredoxin-OBM Fusion Protein (Trx-OBM)

10 µl of 10X ExTaq buffer (TAKARA SHUZO CO., LTD.), 8 µl of 10 mM

dNTPS (TAKARA SHUZO CO., LTD.), 77.5 μl of sterilized distilled water, 2 μl of pOBM291 solution (10 ng/μl), 1 μl of primer OBM3 (100 pmol/μl, SEQ ID NO: 9), 1 μl of primer OBMSalR2 (100 pmol/μl, SEQ ID NO: 10) and 0.5 μl of ExTaq (5μ/μl) (Takara Shuzo Co., Ltd.) were mixed together, and then PCR was conducted in a microtube for centrifugation. After the reaction was carried out at 95°C for 5 minutes, 50°C for 1 second, 55°C for 1 minute, 74°C for 1 second and 72°C for 5 minutes, the cycle reaction consisting of at 96°C for 1 minute, 50°C for 1 second, 55°C for 1 minute, 74°C for 1 second and 72°C for 3 minutes was repeated 25 times. After gel electrophoresis through 1% agarose, an approximately 750 bp DNA fragment was purified from the whole reaction solution with QIAEX® II Gel Extraction Kit (QIAGEN Co., Ltd.). All of the purified DNA fragment was cleaved with restriction enzymes SalI and EcoRI (Takara Shuzo Co., Ltd.), and a DNA fragment about 160-bp (fragment 1) was purified by gel electrophoresis through 1.5% agarose and dissolved in 20 μl of sterilized distilled water. Similarly, 4 μg of pOBM291 and 2 μg of pTrxFus (Invitrogen Co., Ltd.) were cleaved with restriction enzymes BamHI/EcoRI and BamHI/SalI (Takara Shuzo Co., Ltd.), respectively. A DNA fragment about 580-bp (fragment 2) and an approximately 3.6-kb DNA fragment (fragment 3) were purified therefrom, respectively, and dissolved in 20 μl of sterilized distilled water. QIAEX® II Gel Extraction Kit was used for for purifying the fragments. Fragments 1, 2 and 3 were ligated by incubating them using DNA Ligation Kit Ver. 2 (Takara Shuzo Co., Ltd.) at 16°C for 2.5 hours. Then, *E. coli* GI724 cells (Invitrogen Co., Ltd.) were transformed with the ligation product in accordance with the method described in an instruction manual attached to ThioFusion Expression System (Invitrogen Co., Ltd.). Among the resulting ampicillin-resistant transformants, one having a plasmid, in which an OBM cDNA fragment (350-1111 of SEQ ID NO: 2, corresponding to: 76-316 of SEQ ID NO: 1) was linked to a thioredoxin gene in the same reading frame and was selected after analysis of DNA fragment map obtained by restriction enzyme cleavage and DNA sequencing. The obtained strain was referenced as GI724/pTrxOBM25.

(2) Expression of OBM in *E. coli*

The GI724/pTrxOBM25 strain and the GI724 strain having pTrxFus (GI724/pTrxFus) were cultured in 2 ml of RMG-Amp medium (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 1.2% casamino acid (Difco Co., Ltd.), 1% glycerol, 1 mM $MgCl_2$, 100 μg/ml ampicillin (Sigma Co., Ltd.), pH 7.4) with shaking at 30°C for 6 hours. 0.5 ml of the cell suspension was added to 50 ml of Induction medium (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 0.2% casamino acid, 0.5% glucose, 1 mM $MgCl_2$, 100 μg/ml ampicillin, pH 7.4) and cultured with shaking at 30°C. L-

tryptophan was added so that the final concentration was 0.1 mg/ml when the value at OD_{600} became about 0.5, and the cells were further cultured at 30°C for 6 hours. The cell suspension was centrifuged at 3,000 X g and the collected cells were then suspended in 12.5 ml of PBS (10 mM phosphoric acid buffer, 0.15 M NaCl, pH 7.4). The suspension was subjected to ultrasonication using a ultrasonicator (Ultrasonics Co., Ltd.) so that the cells were crushed and then centrifuged at 7,000 X g for 30 minutes. The recovered supernatant was used as soluble protein fraction. 10 μl of the soluble protein fraction solution was subjected to SDS polyacrylamide (10%) electrophoresis under reducing conditions. As a result, a band having a molecular weight of about 40 kDa was observed in the soluble protein fraction solution of GI724/pTrxOBM25, while not observed in soluble protein fraction solution of GI724/pTrxFus (Fig. 14). Thus, it was confirmed that the thioredoxin-OBM fusion protein (Trx-OBM) was expressed in *E. coli*.

(3) Binding Ability of Trx-OBM to OCIF

In the following experiment, it was confirmed that the expressed Trx-OBM bound to OCIF. Anti-thioredoxin antibody (Invitrogen Co., Ltd.) was diluted with 10 mM sodium hydrogencarbonate solution so that the concentration was 1/5,000. 100 μl thereof was added to each well of a 96-well immunoplate (Nunc Co., Ltd.) and then left to stand at 4°C overnight. After the solution in each cell was discarded, 200 μl of 1/2 concentration of BLOCKACE (Snow Brand Milk Products Co., Ltd.) diluted with PBS (BA-PBS) was added to each well and then left to stand at room temperature for 1 hour. After the solution was discarded, 100 μl of the soluble protein fraction solution derived from GI724/pTrxOBM25 which was diluted stepwise with BA-PBS and 100 μl of that derived from GI724/pTrxFus which was diluted stepwise with BA-BPB were added to wells and left to stand at room temperature for 2 hours, respectively. After washing each well three times with PBS-T, 100 μl of OCIF (100 ng/ml) diluted with BA-PBS was added to each well and left to stand at room temperature for 2 hours. After washing each well three times with PBS-T, 100 μl of peroxidase-labeled anti-OCIF rabbit polyclonal antibody desribed in WO 96/26217, which was diluted with BA-PBS so that the concentration was 1/2,000, was added to each well and left to stand at room temperature for 2 hours. After washing each well six times with PBS-T, 100 μl of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added thereto and then left to stand at room temperature for about 10 minutes. Thereafter, 100 μl of Stopping Reagent (Scytek Co., Ltd.) was added thereto. Absorbance of each well at 450 nm was measured with a microplate reader. The results are shown in Fig. 15. When the concentration of the soluble protein fraction solution derived from GI724/pTrxFus increased, the absorbance increased in a concentration (of the added solution)-dependent

manner, while no difference in absorbance was observed between when the soluble protein fraction solution derived from GI724/pTrxFus was added and when said soluble protein fraction solution was not added. Furthermore, Fig. 16 shows the results of experiments when the dilution rate of the soluble fraction solution was kept constant (1%) and OCIF diluted stepwise with BA-PBS (0-100 ng/ml) was further added. Absorbance was kept low regardless of the concentration of OCIF when soluble protein fraction solution derived from GI724/pTrxFus was added. However, absorbance was increased in an OCIF concentration-detendent manner when soluble protein fraction solution derived from GI724/pTrxOBM25 was added. Thus, it was confirmed that the Trx-OBM produced in GI724/pTrxOBM25 had an ability to bind OCIF.

(4) Large Scale Culture of *E. coli* Producing Trx-OBM

GI724/pTrxOBM25 was spread on an RMG-Amp agar medium (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 2% casamino acid, 1% glycerol, 1 mM $MgCl_2$, 100 μg/ml ampicillin, 1.5% agar, pH 7.4) with a platinum loop and cultured at 30°C overnight. The cells were suspended in 10 ml of Induction medium. 5 ml of the suspension was added to two of 2L conical flasks containing 500 ml of Induction medium and cultured by shaking at 30°C. L-tryptophan was added so that the final concentration was 0.1 mg/ml when OD_{600} value became about 0.5, and then the cells were further cultured by shaking at 30°C for 6 hours. The cell suspension was centrifuged at 3,000 X g for 20 minutes, and the cells were collected and then suspended in 160 ml of PBS. The suspension was subjected to ustrasoniaction using an ultrasonicator (Ultrasonics Co., Ltd.) for crushing cells, and then centrifuged at 7,000 X g for 30 minutes. Thereafter, the supernatant was recovered as soluble protein fraction.

(5) Preparation of OCIF-immobilized Affinity Column

2 g of TSKgel AF-Tresyl TOYOPAL 650 (Toso Co., Ltd.) and 40 ml of 1.0 M potassium phosphate buffer (pH 7.5) containing 35.0 mg of recombinant OCIF prepared by a method described in WO 96/26217 were mixed together and gently shaken at 4°C overnight so as to cause a coupling reaction. After the supernatant was removed by centrifugation, 40 ml of 0.1 M Tris-hydrochloric acid buffer (pH 7.5) was added to the precipitated carrier, and the mixture was gently shaken at room temperature for 1 hour, in order to inactivate an excess amount of active groups thereon. After washing the column with both 0.1 M glycine-hydrochloric acid buffer (pH 3.3) containing 0.01% Polysorbate 80/0.2 M NaCl and 0.1 M sodium citrate buffer (pH 2.0) containing 0.01% Polysorbate 80/0.2 M NaCl, the column was washed twice with 10 mM sodium phosphate buffer (pH 7.4) containing 0.01% Polysorbate 80 and equilibrated therewith.

(6) Purification of Trx-OBM Using OCIF-Immobilized Affinity Column

Purification of Trx-OBM was carried out at 4°C unless otherwise stated. The above OCIF-immobilized affinity carrier (10 ml) and the above soluble protein fraction solution (120 ml) described in Example 15-(4) were mixed together. The mixture was gently shaken in four 50 ml centrifugation tubes with a rotor at 4°C overnight. The carrier in the mixture was embeded in Econo-Column (Bio-Rad Co., Ltd., internal diameter: 1.5 cm, length: 15 cm). The column was washed with 300 ml of PBS containing 0.01% Polysorbate 80, 100 ml of 10 mM sodium phosphate buffer (pH 7.0) containing 0.01% Polysorbate 80 and 2 M NaCl and 100 ml of 0.1 M glycine-hydrochloric acid buffer (pH 3.3) containing 0.01% Polysorbate 80 and 0.2 M NaCl. Then, protein was eluted from the column with 0.1 M sodium citrate buffer (pH 2.0) containing 0.01% Polysorbate 80 and 0.2 M NaCl. 5 ml eluate fractions were collected. Immediately, a 10% volume of 2 M Tris solution (pH 8.0) was added for neutralization. The presence or absence of Trx-OBM in each fraction of the eluate was examined in accordance with the above method as described in Example 15-(3) (the ability to bind OCIF). Fractions containing Trx-OBM were collected and further purified.

(7) Purification of Trx-OBM by Gel Filtration

Using Centriplus® 10 and Centricon® 10 (Amicon Co., Ltd.), about 25 ml of the above Trx-OBM fraction of Example 15-(6) was concentrated by centrifugation to a final volume of about 0.5 ml. This sample was subjected to Superose® 12 HR 10/30 column (1.0 X 30 cm, Pharmacia Co., Ltd.) previously equilibrated with PBS containing 0.01% Polysorbate 80. The column was developed using PBS containing 0.01% Polysorbate 80 as a mobile phase at a flow rate of 0.25 ml/min and 0.25 ml eluate fractions were collected from the column. Trx-OBM in the fractions was detected by the method described in Example 15-(3) and by SDS-polyacrylamide electrophoresis (gradient gel of 10 to 15% polyacrylamide, Pharmacia Co., Ltd.) using Phast System (Pharmacia Co., Ltd.) and silver staining. Fractions (Fr. 20 to 23) containing purified Trx-OBM were collected and subjected to measurement of Trx-OBM protein concentration. The measurement was carried out with DC-protein assay kit (Bio-Rad Co., Ltd.) using bovine serum albumin as a standard.

[Example 16]

Osteoclastogenesis Promoting Activity of OBM

COS-7 cells were transfected with pOBM291 and pcDL-SR α296 using lipofectamine (Gibco BRL Co., Ltd.), respectively. After the cells were cultured in DMEM containing 10% FCS for 1 day, they were treated with trypsin, and seeded in a 24-well plate, in which a cover glass (15 mm round, Matsunami Co., Ltd.) was seated, so that the concentration became 5 X 10^4 cells/well. The cells were then cultured for another two days. After washing the culture plate once with PBS, PBS containing 1% paraformaldehyde was added thereto, and the cells were incubated at room temperature for 8 minutes and fixed.

After washing the plate in which the cells were fixed six times with PBS, 700 μl of 1 X 10^6 cells/ml suspension of mouse spleen cell in α-MEM containing 10^{-8} M of the active-form of vitamin D_3, 10^{-7} M dexamethasone and 10% bovine fetal serum was added to each cell. A Millicell® PCF (Millipore Co., Ltd.) was set on each well, and 700 μl of 4 X 10^4 cells/ml suspension of ST2 cells in the above medium was added to the Millicell® PCF and cultured at 37°C for 6 days. After the culture, the Millicell® PCF was removed, and the plate was washed once with PBS. The cells were fixed with acetone-ethanol solution (50:50) for 1 minute, and then only the cells showing tartaric acid resistant acid phosphatase activity (TRAP activity), a specific marker for osteoclasts, were stained with leukocyte acid phosphatase kit (Sigma Co., Ltd.). As a result of observation using a microscope, 45 ± 18 (average ± standard deviation, n = 3) TRAP positive cells were observed in the wells in which pOBM291-transfected COS-7 cells were fixed, while no cells showing TRAP activity were detected in the wells in which pcDL-SR α296-transfected COS-7 cells were fixed. Furthermore, it was also confirmed that calcitonin bound said TRAP positive cells. Thereby, it was revealed that OBM had an activity to promote osteoclast formation.

[Example 17]

Osteoclastogenesis Promoting Activities of Trx-OBM and Secretory-type OBM

Mouse spleen cells were suspended in α-MEM containing 10^{-8} M of the active-form of vitamin D_3, 10^{-7} M dexamethasone and 10% bovine fetal serum in a concentration of 2 X 10^6 cells/ml, and 350 μl of this suspension was added to each well of a 24-well plate. 350 μl of the solution (40 ng/ml) obtained by diluting the purified Trx-OBM with the above medium, 350 μl of solution obtained by diluting the conditioned medium of 293-EBNA cells (which were transduced by pCEP sOBM or pCEP4 cultured in IMDM-10% FCS with the above medium) so that the concentraion was 1/10, or 350 μl of the above medium alone was added to each well. Then, a Millicell® PCF (Millipore Co., Ltd.) was set on each well, and 600 μl of 4 X 10^4 cells/ml suspension of ST2 cell in the above medium were added to the Millicell® PCF. After the cells were cultured for 6 days, the Millicell® PCF was removed, and the plate was washed once with PBS. When the cells were fixed with acetone-ethanol solution (50:50) for 1 minute, only the cells showing tartaric acid resistant acid phosphatase activity (TRAP activity) were stained with LEUKOCYTE ACID PHOSPHATASE kit (Sigma Co., Ltd.). Through observation under a microscope, 106 ± 21 (average ± standard deviation, n = 3) TRAP positive cells were observed in the wells when Trx-OBM was added, while no cells showing TRAP activity were detected in the wells when not added. Similarly, 120 ± 31 (average ± standard deviation, n = 3) TRAP positive cells were observed in the wells when the conditioned medium of 293-EBNA cells transduced by pCEP-sOBM was

added, while no cells showing TRAP activity were detected in the wells when not added. Furthermore, it was confirmed that calcitonin bound to these TRAP positive cells. Thereby, it was revealed that Trx-OBM and secretory-type OBM had an activity to promote osteoclast formation.

[Example 18]

Identity of the Protein OBM Expressed by the cDNA of the Present Invention and Natural-Type OCIF Binding Protein of the Present Invention

(1) Preparation of Anti-OBM Rabbit Polyclonal Antibody

Three male Japanese white rabbits (weight: 2.5 to 3.0 kg, purchased from Kitayama Labeth Co., Ltd.) were subjected to hypodermic immunization with 1 ml of emulsion prepared by mixing 200 μg/ml of purified OBM (thioredoxin-OBM fusion protein), which was obtained in accordance with the methods described in Examples 14-(6) and 14-(7), with 200 μg/ml of Freund's complete adjuvant (Difco Co., Ltd.). The immunization was carried out 6 times in total with one-week interval each, and all the blood was collected from the rabbits on the 10^{th} day counted from the last immunization. An antibody was purified from the fractionated serum in the following manner. That is, the antiserum diluted with PBS to be 1/2 concentraion, and ammonium sulfate was added thereto so that the final concentration was 40% (w/v). Then, the antiserum was left to stand at 4°C for 1 hour and centrifuged at 8,000 X g for 20 minutes. Thereafter, the precipitate was collected and dissolved in a small aliquot of PBS, and then dialyzed against PBS at 4°C. The resulting solution was charged onto a Protein G-Sepharose® column (Pharmacia Co., Ltd.). After washing the column with PBS, immunoglobulin G adsorbed was eluted with 0.1 M glycine-hydrochloric acid buffer (pH 3.0), and the pH thereof was immediately adjusted to be neutral with 1.5 M Tris-hydrochloric acid buffer (pH 8.7). After the eluted protein fraction was dialyzed against PBS, absorbance at 280 nm was measured and its concentration was determined ($E^{1\%}$ 13.5). Horseradish peroxidase-labeled anti-OBM antibody was prepared with Maleimide Activated Peroxidase Kit (Pierce Co., Ltd.). That is, 80 μg of N-succinimide-S-acetylthioacetic acid was added to 1 mg of the purified antibody and allowed to react at room temperature for 30 minutes. 5 mg of hydroxylamine was added thereto for deacetylation, and then the modified antibody was fractionated by using a polyacrylamide desalting column. The protein fraction was mixed with 1 mg of maleimide activated peroxidase and allowed to react at room temperature for 1 hour, and then the enzyme-labeled antibody was obtained.

(2) Inhibition of Specific Binding of the Protein Expressed by the cDNA of the Present Invention (OBM) or Natural-Type Protein of the Present Invention to OCIF by Anti-OBM Rabbit Polyclonal Antibody

2 μg/ml of purified OBM (thioredoxin-OBM fusion protein) obtained in accordance with the methods described in Examples 15-(6) and 15-(7) and 2 μg/ml of natural-type purified OCIF binding protein of Example 2-(4) were dissolved in 0.1 M sodium hydrogencarbonate, respectively. 100 μl of each solution was added to each well of a 96-well immunoplate (Nunc Co., Ltd.) and then left to stand at 4°C overnight. 200 μl of 50% BLOCKACE was added to each well and left to stand at room temperature for 1 hour. After washing wells three times with PBS containing 0.1% Polysorbate 20 (P20-PBS), 200 μg/ml of anti-OBM rabbit antibody was dissolved in 25% BLOCKACE diluted with P20-PBS, and 100 μl of the antibody solution or 25% BLOCKACE without antibody was added to each well and incubated at 37°C for 1 hour. After washing wells three times with P20-PBS, 100 μl of medium for the binding experiment (P20-PBS containing 0.2% bovine serum albumin, 20 mM Hepes and 0.1 mg/ml Heparin) containing 20 ng/ml of the ^{125}I-labeled OCIF described in Example 8-(3) was added thereto. Furthermore, 100 μl of medium for the binding experiment containing 8 μg/ml of unlabeled OCIF in addition to 20 ng/ml of the ^{125}I-labeled OCIF was added to other wells. After incubating the immunoplate at 37°C for 1 hour, each well was washed six times with P20-PBS. The amount of ^{125}I in each well was measured with a gamma counter. The results are shown in Fig. 17. As shown in Fig. 17, neither OBM obtained by expressing the cDNA of the present invention and subsequently purifying or the natural-type protein of the present invention, which specifically binds OCIF, bound the ^{125}I-labeled OCIF when treated with the anti-OBM polyclonal rabbit antibody. On the other hand, it was confirmed that both proteins bound to the ^{125}I-labeled OCIF when not treated with said antibody. Furthermore, it was also revealed that bindings of both proteins to OCIF were specific binding since the bindings were significantly inhibited by addition of a 400-fold higer concentration of unlabeled OCIF (8 μg/ml). From the above results, it was revealed that the anti-OBM rabbit polyclonal antibody recognized both OBM which was a protein expressed by the cDNA of the present invention and the natural-type OCIF binding protein of the present invention, and inhibited specific binding of both proteins to OCIF.

[Example 19]

Cloning of Human OBM cDNA

(1) Preparation of Mouse OBM Primer

For screening of human OBM cDNA, a mouse OBM primer prepared in accordance with the method of the above Example, OBM #3 and OBM #8 were used. Sequences thereof are shown in SEQ ID NO: 9 and SEQ ID NO: 6.

(2) Acquisition of Human OBM cDNA Fragments by PCR

A human OBM cDNA fragment was obtained by PCR method using Human Lymph Node Marathon ready cDNA (Clontech Co., Ltd.) which was a human lymph node derived cDNA library as a mold and using the mouse OBM cDNA primer prepared in the above (1).

The following are the conditions used for PCR.

10X EX Taq buffer (Takara Shuzo Co., Ltd.)	2.0 μl
2.5 mM dNTP	1.6 μl
cDNA solution	1.0 μl
EX Taq (Takara Shuzo Co., Ltd.)	0.2 μl
Distilled Water	14.8 μl
40 μM Primer OBM #3	0.2 μl
40 μM Primer OBM #8	0.2 μl

After the above solutions were mixed together in a microfuge tube, PCR was conducted under the following conditions. A pretreatment was carried out at 95°C for 2 minutes, then the cycle reaction consisting of 95°C for 30 seconds, 57°C for 30 seconds and 72°C for 2.5 minutes was repeated 40 times, and the solution was incubated at 72°C for an approx. 5 minutes. A subfraction of the reaction product and run through agarose by electrophoresis detected an approximate 690 bp DNA fragment amplified with the above mouse OBM cDNA primers.

(3) Purification of Human OBM cDNA Amplified by PCR and Determination of Nucleotide Sequence

The human OBM cDNA fragments obtained in Example 19-(2) were separated by agarose gel electrophoresis and then purified by use of a QIAEX® gel extraction kit (QIAGEN Co., Ltd.). By use of the purified human OBM cDNA fragments as templates, PCR was conducted again by use of the above mouse OBM cDNA primer so as to prepare a large amount of human OBM cDNA fragments which were then purified by use of the QIAEX® gel extraction kit. The nucleotide sequence of the purified human OBM cDNA fragment was determined by use of a Taq Dye Deoxy Terminator Cycle Sequencing FS kit (Perkin Elmer Co., Ltd.) using OBM #3 and OBM #8 (SEQ ID NO: 9 and SEQ ID NO: 6, respectively) as primers. Comparing the nucleotide sequence of the human OBM cDNA fragment with the corresponding part of the mouse OBM cDNA, they share a homology of 80.7%.

(4) Screening for Full Length Human OBM cDNA by Hybridization With Human OBM cDNA Fragments With a Length of About 690 bp as Probes

The human OBM cDNA fragments, with a length of about 690 bp, purified in Example 19-(3) were labeled with [α^{32}P] dCTP by use of a MEGA PRIME DNA labeling kit (Amersham Co., Ltd.), and full length human OBM cDNA was screened. As an object to be screened, a Human Lymph Node 5'-STRETCH PLUS cDNA library (Clontech Co., Ltd., USA) was used. In accordance with a protocol issued by the company, after *Escherichia coli* C600 Hfl was infected with recombinant phage at 37°C for 15 minutes, the *Escherichia coli* was added to an LB agar medium (1% trypton, 0.5% yeast extract, 1% NaCl, 0.7% agar) heated at 45°C and poured onto an LB agar medium plate containing 1.5% agar. After overnight culturing at 37°C, HYBOND® N (Amersham Co., Ltd.) was brought into intimate contact with the plate having plaques formed thereon for about 3 minutes. Then, this filter was subjected to an alkaline denaturation treatment in accordance with a commonly used method, neutralized, and immersed in a 2X SSC solution. The DNA was fixed on the filter by UV CROSSLINK (Stratagene Co., Ltd.). The obtained filter was immersed in a Rapid-hyb buffer (Amersham Co., Ltd.) and pretreated at 65°C for 15 minutes. Thereafter, the filter was transferred into the above buffer containing the above heat denatured human OBM cDNA fragments (about 690 bp, 5 X 10^5 cpm/ml) and allowed to hybridize at 65°C overnight. After the reaction, the filter was washed with 0.1%-SDS-containing 2X SSC once, with 1X SSC once and with 0.1X SSC once in turn at 65°C for 15 minutes. The obtained positive clones were screened two more times so as to purify the clones. A clone having about 2.2 kb of insert was selected out of these and used in the following experiment. The purified phage was named λhOBM. From the purified λhOBM, about 10 μg of DNA was obtained in accordance with a protocol of a QIAGEN® Lambda kit (QIAGEN Co., Ltd.). After this DNA was cleaved with a restriction enzyme SalI, about 2.2 kb of hOBM insert cDNA was separated by agarose electrophoresis. The DNA fragment, purified by use of a QIAEX® gel extraction kit (QIAGEN Co., Ltd.), was cleaved with restriction enzyme SalI in advance and then inserted into dephosphorylated plasmid pUC19 (MBI Co., Ltd.) by use of a DNA ligation kit ver. 2 (Takara Shuzo Co., Ltd.). *Escherichia coli* DH5α (Gibco BRL Co., Ltd.) was transformed by use of the pUC19 containing obtained DNA fragment. The obtained transformant was named pUC19hOBM. After proliferating the transformant, about 2.2 kb of human-OBM cDNA-inserted plasmids were purified thereform in accordance with a commonly used method.

(5) Determination of the Nucleotide Sequence of cDNA Encoding the Full Length Amino Acid Sequence of Human OBM

The nucleotide sequence of the human OBM cDNA obtained in Example 19-(4) was determined by use of a Taq Dideoxy Terminator Cycle Sequencing FS kit (Perkin Elmer Co., Ltd.). That is, the nucleotide sequence of the inserted fragment was determined by use

of pUC19hOBM as a template. M13 Primer M3, M13 Primer RV (TAKARA SHUZO CO., LTD.), and a synthetic primer human OBM #8 designed based on the nucleotide sequence of the human OBM cDNA fragment (about 690 bp) were used as primers for determining the nucleotide sequence of the inserted fragment DNA of pUC19. The sequences of the primers, M13 Primer M3 and M13 Primer RV, are shown in SEQ ID NO: 4 and SEQ ID NO: 5, respectively. The amino acid sequence of human OBM estimated from the nucleotide sequence of the human OBM cDNA is shown in SEQ ID NO: 11, and the nucleotide sequence of the human OBM cDNA is shown in SEQ ID NO: 12.

The obtained plasmid containing the human OBM cDNA and the obtained *Escherichia coli* transformed by pUC19hOBM were deposited with the National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry with the deposition number FERM BP-6058 on August 13, 1997.

[Example 20]

^{125}I Labeling of OCIF and Quantitative Determination of ^{125}I-Labeled OCIF by ELISA

OCIF was ^{125}I-labeled in accordance with Iodogen method. 20 µl of 2.5 mg/ml Iodogen-chloroform solution was transferred to a 1.5 ml Eppendorf tube, and chloroform was evaporated at 40°C so as to prepare an Iodogen-coated tube. After the tube was washed with 400 µl of 0.5 M sodium phosphate buffer (Na-Pi; pH 7.0) three times, 5 µl of 0.5 M Na-Pi with a pH of 7.0 was added. Immediately after 1.3 µl (18.5 MBq) of Na-^{125}I solution (Amersham Co., Ltd., NEZ-033H) was added to the tube, 10 µl of 1 mg/ml OCIF solution (monomer type or dimmer type) was added. The resulting solution was mixed by means of a vortex mixer and left to stand at room temperature for 30 seconds. The solution was transferred to a tube containing 80 µl of 0.5 M Na-Pi solution (pH 7.0), which contained 10 mg/ml potassium iodide and 5 µl of phosphate buffered saline containing 5% bovine serum albumin (BSA-PBS), and mixed. The solution was added to a spin column (1 ml, G-25 Sephadex® fine, Pharmacia Co., Ltd.) equilibrated with BSA-PBS and centrifuged at 2,000 rpm for 5 minutes. After 400 µl of BSA-PBS was added to a fraction eluted from the column and the fraction was mixed, 2 µl of the each fraction was sampled, and the radioactivity of the sample was measured by means of a gamma counter. The radiochemical purity of the prepared ^{125}I labeled OCIF solution was determined by measuring the radioactivity of a fraction precipitated by addition 10% trichloroacetic acid (TCA).

The OCIF biological activity of the ^{125}I labeled OCIF was measured in accordance with a method described in WO 96/26217. Further, the concentration of ^{125}I labeled OCIF was measured by ELISA in the following manner. That is, 100 µl of 50 mM

$NaHCO_3$ (pH 9.6), having 2 μg/ml of rabbit anti-OCIF polyclonal antibody described in WO 96/26217 dissolved therein, was added to each well of 96-well immunoplate (Nunc Co., Ltd., MaxiSorp) and left to stand at 4°C overnight. After this solution was discarded, 200 μl of mix-solution of BLOCKACE (Snow Brand Milk Products Co., Ltd.) and a phosphate buffeed saline (mixing ratio = 25:75) (B-PBS) was added to each well and then left to stand at room temperature for 2 hours. After the solution was discarded, each well was washed with a phosphate buffered saline containing 0.01% Polysorbate 80 (P-PBS) three times. Thereafter, 100 μl of B-PBS containing a ^{125}I labeled OCIF sample or OCIF reference standard was added to each well and left to stand at room temperature for 2 hours. After the solution was discarded, each well was washed with 200 μl of P-PBS six times. Then, 100 μl of diluted solution of peroxidase-labeled anti-OCIF rabbit polyclonal antibody in B-PBS was added to each well and left to stand at room temperature for 2 hours. After the solution was discarded, each well was washed with 200 μl of P-PBS six times. Then, 100 μl of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added to each well and then left to stand at room temperature for 2 to 3 minutes. Thereafter, 100 μl of Stopping Reagent (Scytek Co., Ltd.) was added to each well. The absorbance of each well at 450 nm was measured by means of a microplate reader. The concentration of the ^{125}I labeled OCIF was determined from a calibration curve prepared by use of the OCIF reference standard.

[Example 21]

Expression of Protein Encoded by the cDNA of the Present Invention

(1) Construction of hOBM Expression Vector for Animal Cell

pUChOBM was cleaved with restriction enzyme SalI, and about 2.2 kb DNA fragments were purified by 1% agarose gel electrophoresis and blunt-ended with DNA Blunting Kit (Takara Shuzo Co., Ltd.) (the resulting DNA fragment with smoothed terminals is called "smoothed hOBM cDNA fragment"). Expression plasmid pcDL-SR α296 (Molecular and Cellular Biology, Vol. 8, pp. 466 to 472 (1988)) was cleaved with a restriction enzyme EcoRI, and blunt-ended with the blunting kit. The resulted expression plasmid was bound to the smoothed hOBM cDNA fragment by use of a DNA ligation kit ver. 2. Using the ligation reaction solution, *Escherichia coli* DHα was transformed. From the obtained ampicillin-resistant transformant, a clone, having a phOBM plasmid in which hOBM cDNA inserted with forward direction for transcription direction of SRα promoter, was selected by analysis of DNA map obtained by restriction enzyme cleavage and determination of DNA sequences. The obtained clone was named DH5α/phOBM.

(2) Expression of Human OBM in COS-7 Cell

E coli, DH5α/phOBM, was cultured and the plasmid phOBM was purified with

QIA® Filter Plasmid Midi Kit (QIAGEN Co., Ltd.). The phOBM was transfected into COS-7 cells in each well of 6 well plate by use of lipofectamine, and the cells were cultured in DMEM containing 10% fetal bovine serum for 2 days. The medium was replaced with cysteine/methionine-free DMEM (Dainippon Pharmaceutical Co., Ltd.) containing 5% dialyzed fetal bovine serum (88 μl/well), and the cells were cultured for another 15 minutes. Then, 14 μl of Express Protein Labeling Mix (NEN Co., Ltd., 10 mCi/ml) was added. After the cells were cultured for 4 hours, 200 μl of DMEM containing 10% fetal bovine serum was added, and the cells were cultured for 1 hour. After the cells were washed with PBS twice, 0.5 ml of TSA buffer (10 mM Tris-HCl (pH 8.0) containing 0.14 M NaCl and 0.025% NaN_3) containing 1% Triton X-100, 1% bovine hemoglobin, 10 μg/ml leupeptin, 0.2 TI U/ml aprotinin and 1 mM PMSF was added, and the cells were left to stand on ice for 1 hour. After the cells were crushed by pipetting, the resulting lysate was centrifuged at 4°C and 3,000 X g for 10 minutes so as to obtain a supernatant. 200 μl of dilution buffer (TSA buffer containing 0.1% Triton X-100, 0.1% bovine hemoglobin, 10 μg/ml leupeptin, 0.2 TIU/ml aprotinin and 1 mM PMSF) was added to 100 μl of the supernatant, and the resulting supernatant was shaken together with Protein A Sepharose® (50 μl) at 4°C for 1 hour. Thereafter, the solution was centrifuged at 4°C, 1,500 X g for 1 minute so as to collect a supernatant. Thereby, a protein non-specifically binding the Protein A Sepharose® was removed. OCIF (1 μg) was added to the supernatant, and the resulting supernatant was shaken at 4°C for 1 hour so as to bind OBM and OCIF together. Then, an anti-OCIF rabbit polyclonal antibody (50 μg) was added, and the resulting solution was shaken at 4°C for 1 hour. Then, Protein A Sepharose® (10 μl) was added to the solution and the solution was then shaken at 4°C for 1 hour. The solution was centrifuged at 4°C, 1,500 X g for 1 minute so as to collect a precipitated fraction. The precipitate resulting from the centrifugation was washed with the dilution buffer twice, with a bovine hemoglobin free dilution buffer twice, with a TSA buffer once, and with 50 mM Tris-HCl (pH 6.5) once. After washing, an SDS buffer (0.125 M Tris-HCl, 4% dodecyl sodium sulfate, 20% glycerol, 0.002% bromophenol blue, pH 6.8) containing 10% β-mercaptoethanol was added to the precipitate. The precipitate was heated at 100°C for 5 minutes, and it was subjected to SDS-PAGE (12.5% polyacrylamide gel, Daiichi Kagaku Co., Ltd.). The gel was fixed and dried in accordance with a commonly used method, and the signals of isotopes from the fixed gel were amplified by Amplify® (Amersham Co., Ltd.). The fixed gel was exposed to BioMax® MR Film (Kodak Co., Ltd.) at -80°C. The results are shown in Fig. 8. As a result, it was revealed that the molecular weight of protein encoded by the cDNA of the present invention was about 40,000.

[Example 22]

Binding of Protein Encoded by the cDNA of the Present Invention to OCIF

In the same manner as in Example 21-(2), the purified phOBM was transfected into COS-7 cells in each well of a 24 well plate by the use of lipofectamine, and the cells were cultured for 2 or 3 days. Then, the cells were washed with serum-free DMEM, and 200 μl of medium for the binding assay (serum-free DMEM containing 0.2% bovine serum albumin, 20 mM Hepes buffer, 0.1 mg/ml heparin and 0.2% NaN_3), containing 20 ng/ml of ^{125}I labeled OCIF was added to some wells. In addition, to other wells, 200 μl of the medium for binding assay, containing 8 μg/ml of unlabeled OCIF in addition to 20 ng/ml of the ^{125}I labeled OCIF, was added so as to conduct following experiments. After culture in a CO_2 incubator (5% CO_2) at 37°C for 1 hour, the cells were washed twice with 500 μl of phosphate buffered saline containing 0.1 mg/ml heparin. After washing, 500 μl of 0.1 N NaOH solution was added to each well, and the wells were then left to stand at room temperature for 10 minutes so as to dissolve the cells. The amount of ^{125}I in each well was measured by means of a gamma counter. As a result, it was confirmed that the ^{125}I labeled OCIF bound only to a cell transfected with phOBM as shown in Fig. 19. Further, it was also confirmed that the binding was significantly inhibited by addition of a 400-fold concentration of unlabeled OCIF (8 μg/ml). From these results, it was revealed that a human OBM protein, coded for by a cDNA on phOBM, specifically bound to OCIF on the surface of a COS-7 cell.

[Example 23]

Crosslinking Experiment of ^{125}I Labeled OCIF to Protein Encoded by the cDNA of the Present Invention

To further analyze the characteristics of the protein encoded by the cDNA of the present invention, crosslinking of ^{125}I labeled monomer type OCIF with the protein encoded by the cDNA of the present invention was conducted. That is, after expression vectors phOBM were prepared and transfected into COS-7 cells in accordance with the methods described in Examples 21-(1) and (2), 200 μl of the medium for the binding assay containing the ^{125}I labeled OCIF (25 ng/ml) was added to some wells. In addition, the medium for the binding assay, containing unlabeled OCIF of a 400-fold concentration in addition to the ^{125}I labeled OCIF, was added to other wells. The cells were cultured in a CO_2 incubator (5% CO_2) at 37°C for 1 hour, and the cells were washed twice with 500 μl of phosphate buffered saline containing 0.1 mg/ml of heparin. To these cells, 500 μl of phosphate buffered saline containing 100 μg/ml of crosslinking agent DSS (Disuccinimidyl suberate, Pierce Co., Ltd.) was added, and the cells incubated at 0°C for 10 minutes for reaction. After the cells in these wells were washed twice with 1 ml of phosphate buffered saline cooled to 0°C, 100 μl of 20

mM Hepes buffer containing 1% Triton X-100 (Wako Pure Chemical Industries, Ltd.), 2 mM PMSF (phenylmethylsulfonyl fluoride, Sigma Co., Ltd.), 10 μM pepstatin (Wako Pure Chemical Industries, Ltd.), 10 μM leupeptin (Wako Pure Chemical Industries, Ltd.), 10 μM antipain (Wako Pure Chemical Industries, Ltd.) and 2 mM EDTA (Wako Pure Chemical Industries, Ltd.) were added to these cells, and the wells were left to stand at room temperature for 30 minute so as to lyse the cells. After 15 μl of these samples were treated with SDS under nonreducing conditions in accordance with a commonly used method, the samples were subjected to electrophoresis with a gel for SDS electrophoresis (4 to 20% polyacrylamide gradient, Daiichi Kagaku Co., Ltd.). After the electrophoresis, the gel was dried and exposed to BioMax® MS Film (Kodak Co., Ltd.) with BioMax® MS Intensifying Amplifying Screen (Kodak Co., Ltd.) at -80°C for 24 hours. The exposed films were developed in accordance with a commonly used method. As a result, a protein band having a molecular weight of about 90,000 to 110,000 was detected as shown in Fig. 20 by crosslinking between ^{125}I labeled monomer type OCIF and the protein encoded by the cDNA of the present invention.

[Example 24]

Expression of Secretory-Type Human OBM

(1) Construction of Secretory-Type Human OBM Expressing Plasmid

A PCR reaction was carried out by use of human OBM SF (SEQ ID NO: 13) and mouse OBM #8 (SEQ ID NO: 6) as primers and pUC19hOBM as a template. After the product was purified by agarose gel electrophoresis, it was cleaved with restriction enzymes SpI and HindIII and then purified by agarose gel electrophoresis so as to obtain 0.27 kb fragment. A fragment of hOBM cDNA which was cleaved at only one site of restriction enzyme DraI by partial cleavage of human OBM cDNA therewith, and purified by agarose gel electrophoresis, and the purified fragment was further cleaved with a restriction enzyme HindIII. 0.53 kb of DraI/HindIII fragment was purified by agarose gel electrophoresis, and the purified fragment and the SpI/HindIII fragment (0.27 kb) of the above PCR product together with an SpI/EcoRV fragment (5.2 kb) of pSec TagA (Invitrogen Co., Ltd.) were subjected to ligation by use of a ligation kit ver. 2 (TAKARA SHUZO CO., LTD.), and *Escherichia coli* DH5α were transformed by use of the reaction product of ligation. Plasmids were purified from the obtained ampicillin-resistant clone by alkaline SDS method and cleaved by restriction enzymes so as to select a plasmid having 0.27 kb and 0.53 kb of fragments inserted in pSec TagA. The plasmid was subjected to sequencing by use of a Taq Dideoxy Terminator Cycle Sequencing FS Kit (Perkin Elmer Co., Ltd.), thereby confirming that the plasmid had sequences encoding secretory-type human OBM. After the plasmid was cleaved by restriction enzymes NheI and XhoI, a fragment (0.8 kb) corresponding to

secretory-type human OBM cDNA was collected by agarose gel electrophoresis. This fragment was inserted into an NheI/XhoI fragment (10.4 kb) of an expression vector pCEP4 (Invitrogen Co., Ltd.) by use of the ligation kit, and *Escherichia coli* DH5α were transformed by use of the reaction product of the ligation. Plasmids were purified from the obtained ampicillin-resistant clones by alkaline SDS method and cleaved by restriction enzymes so as to select a *Escherichia coli* clone having a secretory-type human OBM expression plasmid (pCEPshOBM) with a target structure. The *Escherichia coli* clone having the pCEPshOBM was cultured, and the pCEPshOBM was purified by use of QIA® Filter Plasmid Midi Kit (QIAGEN Co., Ltd.).

(2) Expression of Secretory-Type OBM

293-EBNA cells were suspended in IMDM containing 10% FCS (IMDM-10%FCS), seeded in a collagen-coated 24 well plate (Sumitomo Bakelite Co., Ltd.) in an amount of 2 X 10^5 cells/2 ml/well, and cultured overnight. To the cells, 1 μg of pCEPshOBM or pCEP4 was transfected by use of 4 μl of lipofectamine (Gibco Co., Ltd.), and the cells were cultured for another 2 days in 0.5 ml of serum-free IMDM or IMDM-10%FCS, thereby collecting a conditioned medium. Expression of secretory-type human OBM in the conditioned medium was confirmed in the following manner. That is, sodium hydrogen carbonate was added to the conditioned medium to a final concentration of 0.1 M and left to stand at 4°C overnight, and the human OBM in the conditioned medium was solid-phased in a 96 well plate. BLOCKACE (Snow Brand Milk Products Co., Ltd.) solution diluted 4 times with PBS (B-PBS) was added to each well and the plate was left to stand at room temperature for 2 hours to cause blocking. 3-100 ng/ml of OCIF diluted with B-PBS was added to the wells and left to stand at 37°C for 2 hours. After the plate was washed with PBS containing 0.05% Polysorbate 20 (P-PBS), 100 μl of peroxidase labeled anti-OCIF antibody described in WO 96/26217 diluted with B-PBS was added to each well and left to stand at 37°C for 2 hours. After each well was washed with P-PBS six times, 100 μl of TMB solution (TMB Soluble Reagent, High Sensitivity, Scytek Co., Ltd.) was added to each well and then left to stand at room temperature for about 10 minutes. Thereafter, 100 μl of Stopping Reagent (Scytek Co., Ltd.) was added to each well. The absorbance of each well at 450 nm was measured by means of a microplate reader. The results are shown in Fig. 21. In the plate having the solid-phased conditioned medium of the cells transfected with the pCEPshOBM, absorption at 450 nm increased depending on the concentration of the OCIF added. Meanwhile, in the case where the conditioned medium of the cells transfected only with the vector pCEP4 was solid-phased, no increase in absorption was seen. Further, Fig. 22 shows the results of an experiment in which the proportion of the conditioned medium used

for solid phasing was varied within a range of 5 to 90% and a certain concentration of OCIF (50 ng/ml) was added. In the plate having the solid-phased conditioned medium of the cells transfected with the pCEPshOBM, absorption at 450 nm increased along with an increase in the proportion of the conditioned medium added. Meanwhile, in the plate having the solid-phased conditioned medium of the cells transfected with the vector pCEP4, no increase in absorption was observed. From these results, it was confirmed that secretory-type human OBM was expressed in the conditioned medium of the cells transfected with the pCEPshOBM.

[Example 25]

Expression of Thioredoxin-Human OBM Fusion Protein (Trx-hOBM)

(1) Construction of Thioredoxin-Human OBM Fusion Protein (Trx-hOBM) Expression Vector

10 μl of 10X ExTaq buffer (Takara Shuzo Co., Ltd.), 8 μl of 10 mM dNTP (Takara Shuzo Co., Ltd.), 77.5 μl of sterilized distilled water, 2 μl of pUC19hOBM aqueous solution (10 ng/μl), 1 μl of primer mouse OBM #3 (SEQ ID NO: 9) (100 pmol/μl), 1 μl of primer hOBM SalR2 (SEQ ID NO: 14) (100 pmol/μl) and 0.5 μl of ExTaq (5μ/μl) (Takara Shuzo Co., Ltd.) were mixed together in a microcentrifuge tube so as to cause a PCR reaction. After the reaction consisiting of 95°C for 5 minutes, 50°C for 1 second, 55°C for 1 minute, 74°C for 1 second and 72°C for 5 minutes, the cycle reaction consisting of at 96°C for 1 minute, 50°C for 1 second, 55°C for 1 minute, 74°C for 1 second and 72°C for 3 minutes, was repeated 25 times. An approximately 750 bp DNA fragment was purified from the whole reaction solution. After the purified DNA fragment (whole) was cleaved with restriction enzymes SaI (TAKARA SHUZO CO., LTD.) and BspHI (NEW ENGLAND BILABS CO., LTD.), 1% agarose gel electrophoretic migration was carried out so as to purify an approximately 320 bp DNA fragment (fragment 1) and dissolve the fragment in 20 μl of sterilized distilled water. Similarly, an approximately 450 bp DNA fragment (fragment 2) which is a cleaved product of 4 μg of pUC19hOBM described in Example 19-(3) by a restriction enzyme BamHI and BspHI (TAKARA SHUZO CO., LTD.) and about 3.6 kb of DNA fragment (fragment 3) which is a cleaved product of 2 μg of pTrXFus (Invitrogen Co., Ltd.) by a restriction enzyme BamHI and SaI (TAKARA SHUZO CO., LTD.) were purified and then dissolved in 20 μl of sterilized distilled water. To purify the DNA fragments, a QIAEXR II gel extraction kit was used. Fragment 1, 2 and 3 were combined by use of a DNA ligation kit ver. 2 (TAKARA SHUZO CO., LTD.) by keeping them at 16°C for 2.5 hours. *Escherichia coli* GI724 strain (Invitrogen Co., Ltd.) was transformed using the ligation reaction solution, in accordance with a method described in an instruction manual

attached to a ThioFusion Expression System (Invitrogen Co., Ltd.). From the obtained ampicillin-resistant transformants, a clone, having a plasmid in which an hOBM cDNA fragment was bound to a thioredoxin gene in the same reading frame, was selected by analysis of DNA mapping obtained by restriction enzyme cleavage and determination of DNA sequences. The obtained strain was named GI724/pTrxhOBM25.

(2) Expression of Trx-OBM in *Escherichia coli*

A GI724/pTrxhOBM strain and a GI724 strain transformed with pTrxFus (GI724/pTrxFus) were cultured shaking at 37°C for 6 hours in 2 ml of RMG-Amp medium (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 2% casamino acid, 1% glycerol, 1 mM $MgCl_2$, 100 μg/ml ampicillin, pH 7.4). 0.5 ml of the culture suspension was added to 50 ml of Induction medium (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 0.2% casamino acid, 0.5% glucose, 1 mM $MgCl_2$, 100 μg/ml ampicillin, pH 7.4) and cultured shaking at 30°C. L-tryptophan was added so as to achieve a final concentration of 0.1 mg/ml when the value at $OD_{600\ nm}$ became about 0.5, and the cells were further shaking-cultured at 30°C for another 6 hours. The cculture suspension was centrifuged at 3,000 X g so as to collect cells, and then the collected cell was suspended in 12.5 ml of PBS. The suspension was subjected to an ultrasonic generator (Ultrasonics Co., Ltd.) so as to crush the cells. Then the sample was centrifuged at 7,000 X g for 30 minutes so as to collect a soluble protein fraction as a supernatant. 10 μl of the solution fraction was subjected to SDS-PAGE (10% polyacrylamide) under reducing conditions. As a result, shown in Fig. 23, a protein band having a molecular weight of about 40,000, which could not be seen in the soluble protein fraction of GI724/pTrxFus, was detected in the soluble protein fraction of GI724/pTrxOBM. From the above results, it was confirmed that a thioredoxin-human OBM fusion protein (Trx-OBM) was expressed in the Echerichia coli clone.

(3) Binding Ability of Trx-hOBM *Escherichia coli* to OCIF

It was confirmed by the following experiment that the expressed Trx-hOBM bound to OCIF. That is, 100 μl of anti-thioredoxin antibody (Invitrogen Co., Ltd.) diluted to be 1/5,000 with 10 mM sodium hydrogen carbonate aqueous solution was added to each well of a 96 well immunoplate (Nunc Co., Ltd.), and the plate was left to stand at 4°C overnight. After the solution in each cell was discarded, 200 μl of a solution obtained by diluting BLOCKACE (Snow Brand Milk Products Co., Ltd.) to be 1/2 with PBS (BA-PBS) was added to each well, and then the plate was left to stand at room temperature for 1 hour. After the solution was discarded, each well was washed with P-PBS three times. 100 μl of the GI724/pTrxOBM-derived soluble protein fraction solution diluted stepwise with BA-PBS, and 100 μl of the GI724/pTrxFus-derived soluble protein fraction solution diluted stepwise

with BA-PBS, were added to each well and the plate was left to stand at room temperature for 2 hours. After each well was washed with P-PBS three times, 100 μl of OCIF (100 ng/ml) diluted with BA-PBS, was added to each well and the plate was left to stand at room temperature for 2 hours. After each well was washed with P-PBS three times, 100 μl of peroxidase-labeled anti-OCIF antibody described in WO 96/26217, diluted to be 1/2,000 with BA-PBS, was added to each well, and the plate was left to stand at room temperature for 2 hours. After each well was washed with P-PBS six times, 100 μl of TMB solution was added to each well, and then the plate was left to stand at room temperature for about 10 minutes. Thereafter, 100 μl of Stopping Reagent was added to each well. The absorbance of each well at 450 nm was measured by means of a microplate reader. The results are shown in Fig. 24. No difference was observed between absorbance resulted in the presence and absence of the GI724/pTrxFus-derived soluble protein fraction solution and, while with the GI724/pTrxhOBM-derived soluble protein fraction solution, the absorbance increased depending on an increase in the concentration of the GI724/pTrxOBM derived soluble protein fraction solution. Further, Fig. 25 shows the results of an experiment in which the dilution rate of the soluble protein fraction solution to be added was kept constant (1% concentration) and OCIF (0-100 ng/ml) diluted stepwise with BA-PBS was added. For the GI724/pTrxFus-derived soluble protein fraction solution, absorbance remained low regardless of the concentration of OCIF, while for the GI724/pTrxhOBM-derived soluble protein fraction solution, absorbance increased in OCIF concentration-dependent manner. It was confirmed from this result that Trx-hOBM produced in GI724/pTrxhOBM had an ability to bind OCIF.

(4) Large Scale Culture of *Escherichia coli* Producing Trx-hOBM

GI724/pTrxhOBM was spread on an RMG-Amp agar medium (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 2% casamino acid, 1.5% agar, pH 7.4) with a platinum loop and cultured at 30°C overnight. The cells were suspended in 10 ml of Induction medium, and every 5 ml of the suspension was added to each of two conical flasks of 2L volume containing 500 ml of Induction medium, and the flasks were shaking-cultured at 30°C. L-tryptophan was added so as to achieve a final concentration of 0.1 mg/ml when absorbance at $OD_{600\,nm}$ became about 0.5, and the shaking culture at 30°C was continued for another 6 hours. The culture suspension was centrifuged at 3,000 X g for 20 minutes so as to collect cells and the collected cells were then suspended in 160 ml of PBS. The suspension was subjected to ultrasocination (Ultrasonics Co., Ltd.) so as to crush the cells, and the cell lysate was then centrifuged at 7,000 X g for 30 minutes so as to collect a soluble protein fraction as a supernatant.

(5) Preparation of OCIF-Immobilized Affinity Column

2 g of TSKgel AF-Tresyl TOYOPAL 650 (TOSO CO., LTD.) and 40 ml of 1.0 M potassium phosphate buffer (pH 7.5) containing 35.0 mg of recombinant OCIF, prepared by a method described in WO 96/26217, were mixed together and gently shaken at 4°C overnight so as to cause a coupling reaction. To inactivate excessive active residue, after a supernatant was removed by centrifugation, 40 ml of 0.1 M Tris-HCl buffer (pH 7.5) was added to a precipitated carrier, and the mixture was gently shaken at room temperature for 1 hour. After 0.1 M glycine-HCl buffer containing 0.01% Polysorbate 80 and 0.2 M NaCl (pH 3.3) and a 0.1 M sodium citrate buffer containing 0.01% Polysorbate 80 and 0.2 M NaCl (pH 2.0) were passed through a column (in which the obtained gel was packed) so as to wash it, the column was washed twice with 10 mM sodium phosphate buffer containing 0.01% Polysorbate 80 (pH 7.4) so as to equilibrate it.

(6) Purification of Trx-hOBM by OCIF-Immobilized Affinity Column

Purification of Trx-hOBM was carried out at 4°C unless otherwise stated. After the above-mentioned OCIF-immobilized affinity carrier (10 ml) and the above-mentioned soluble protein fraction solution (120 ml) described in Example 25-(4) were mixed together, the mixture was gently shaken at 4°C overnight in four 50 ml centrifuge tubes by use of a rotor. The carrier in the mixture was filled an EconoColumn (internal diameter: 1.5 cm, length: 15 cm, Bio-Rad Co., Ltd.). 300 ml of PBS containing 0.01% Polysorbate 80, 100 ml of 10 mM phosphate buffer containing 0.01% Polysorbate 80 and 2.0 M NaCl (pH 7.0), and 100 ml of 0.1 M glycine-HCl buffer containing 0.01% Polysorbate 80 and 0.2 M NaCl (pH 3.3) were passed through the column, in turn, so as to wash the column. Then, 0.1 M sodium citrate buffer containing 0.01% Polysorbate 80 and 0.2 M NaCl (pH 2.0) was passed through the column so as to elute proteins adsorbed to the column. 5 ml eluates were fractionated. To the fractions, 10% volume of 2M Tris solution (pH 8.0) was added so as to immediately neutralize the fractions. The presence or absence of Trx-hOBM in the each fraction of the eluate was examined in accordance with the method described in Example 25-(3). Fractions containing Trx-hOBM were collected and purified further.

(7) Purification of Trx-hOBM by Gel Filtration

About 25 ml of the Trx-hOBM fraction described in Example 25-(6) was concentrated using a centrifuge to about 0.5 ml by use of a Centriplus R10 and a Centricon R10 (Amicon Co., Ltd.). The concentrated sample was subjected to a Superose R12 HR 10/30 column (1.0 X 30 cm, Pharmacia Co., Ltd.) equilibrated in advance with PBS containing 0.01% Polysorbate 80. The column was developed at a flow rate of 0.25 ml/min by using PBS containing 0.01% Polysorbate 80 as a mobile phase so as to fractionate every 0.25 ml of eluates from the column. Trx-OBM in the fractions was detected by the method described in Example 25-(3) and SDS-PAGE. Fractions containing purified Trx-hOBM were

collected so as to measure the protein concentration of Trx-OBM. The protein concentration was measured with DC-protein assay kit (Bio-Rad Co., Ltd.) using bovine serum albumin as a reference standard.

[Example 26]

Osteoclastogenesis Inducing Activity of OBM

phOBM and pcDL-SRα296 were transfected into COS-7 cells by use of lipofectamine (Gibco Co., Ltd.), respectively. After the cells were cultured in DMEM containing 10% FCS for 1 day, they were trypsinized and seeded in a 24-well plate, in which glass cover slips (15 mm round, Matsunami Co., Ltd.) were seated, at a concentration of 5X 10^4 cells/well and then cultured for another two days. The culture plate was washed with PBS once and then PBS containing 1% paraformaldehyde was added, and the cells were incubated at room temperature for 8 minutes so as to fix the cells on the glass cover slips. After the plate with fixed cells was washed with PBS six times, 700 μl of mouse spleen cells suspended in α-MEM (containing 10^{-8} M activated vitamin D_3, 10^{-7} M dexamethasone and 10% fetal bovine serum) in an amount of 1 X 10^6 cells/ml were added to each well. A MILLICELL® PCF (Millipore Co., Ltd.) was set on each well, and 700 μl of ST2 cells, suspended in the above medium in a concentration of 4 X 10^4 cells/ml were added to the MILLICELL® PCF and cultured at 37°C for 6 days. After that, the MILLICELL® PCF was removed and the plate was washed with PBS once. Then, the cells were fixed for a minute by an acetone-ethanol solution (50:50), and cells, having tartaric acid resistant acid phosphatase activity (TRAP activity), which is specific marker of osteoclast, were stained by use of a leukocyte acid phosphatase kit (Sigma Co., Ltd.). Using a microscope, cells having TRAP activity were not detected in the wells having COS-7 cells transfected with the pcDL-SR α296, while 65 ± 18 (n = 3, average ± standard deviation) of TRAP positive cells were observed in the wells having cells transfected with phOBM. Further, it was also confirmed that these TRAP positive cells expressed calcitonin receptors, since the cells showed specific binding to ^{125}I-labeled salmon calcitonin (Amersham Co., Ltd.). From these results, it was revealed that human OBM, a protein encoded by the cDNA of the present invention, had an activity to promote osteoclast formation.

[Example 27]

Osteoclastogenesis Promoting Activities of Trx-hOBM and Secretory-Type Human OBM

Mouse spleen cells were suspended in α-MEM containing 10^{-8} M activated vitamin D_3, 10^{-7} M dexamethasone and 10% fetal bovine serum at a concentration of 2 X 10^6 cells/ml, and 350 μl of the suspension was added to each well of a 24 well plate. After 350

μl of a solution prepared by diluting purified Trx-OBM,(40 ng/ml) with the above medium, 350 μl of a solution prepared by diluting a conditioned medium obtained when 293-EBNA cells transduced by pCEPshOBM or pCEP4 were cultured in IMDM-10%FCS to be 1/10 with the above medium, or 350 μl of the above medium alone was added, a MILLICELL® PCF (Millipore Co., Ltd.) was set on each well, and 600 μl of ST2 cell suspension in the above medium at a concentration of 4 X 10^4 cells/ml were added to the Millicell® PCF. After the cell were cultured for 6 days, the Millicell® PCF was removed, and the plate was washed with PBS once. Then, after the cells were fixed for 1 minute by an acetone-ethanol solution (50:50), cells having tartaric acid resistant acid phosphatase activity (TRAP activity) were stained by use of a leukocyte acid phosphatase kit (Sigma Co., Ltd.). Using a microscope, cells having TRAP activity were not detected in the wells not containing Trx-hOBM, while 115 ± 19 (n = 3, average ± standard deviation) of TRAP positive cells were observed in the wells containing Trx-hOBM. Similarly, cells having TRAP activity were not detected in the wells containing the conditioned medium of pCEP4-transfected 293-EBNA, while 125 ± 23 (n = 3, average ± standard deviation) of TRAP positive cells were observed in the wells containing the conditioned medium of pCEPshOBM-transfected 293-EBNA. Furthermore, it was also confirmed that these TRAP positive cells expressed calcitonin receptors, since the cells showed specific binding to ^{125}I labeled sermon calcitonin (Amersham Co., Ltd.). From these results, it was revealed that Trx-hOBM and secretory-type OBM had an activity to promote osteoclast formation.

[Example 28]

Preparation of Polyclonal Antibody

Mouse sOBM or human sOBM, which was used as an immunizing antigen, was obtained in accordance with the above-mentioned method. That is, mouse sOBM cDNA (cDNA which encodes mouse sOBM (SEQ ID NO: 16) having no membrane binding site and lacking amino acids between the N-terminal end and amino acid 72 of mouse OBM; SEQ ID NO: 18) or human OBM cDNA (cDNA which encodes human sOBM (SEQ ID NO: 17) having no membrane binding site and lacking amino acids region between the N terminal end and amino acid 71 of human OBM; SEQ ID NO: 19), together with a Hind III/EcoRV fragment (5.2 kb) of a pSec TagA expression vector (Invitrogen Co., Ltd.), containing nucleotide sequence coding a signal peptide of κ-chain of immunoglobulin, and an EcoRI/PmaCI fragment (0.32 kb) of OBM cDNA, were subjected to ligation by use of a ligation kit ver. 2 (TAKARA SHUZO CO., LTD.). *Escherichia coli* DH5α were transformed with the reaction product. Plasmids were purified from the obtained ampicillin-resistant clones by alkaline SDS method and cleaved by restriction enzymes so as to select a plasmid

having 0.6 kb and 0.32 kb of fragments inserted in pSec TagA. As a result of determining the sequences of the plasmid by use of Dye Terminator Cycle Sequencing FS kit (Perkin Elmer Co., Ltd.), it was confirmed that the plasmid had sequences encoding mouse or human sOBM. The plasmid was cleaved by restriction enzymes NheI and XhoI and then a fragment (1.0 kb) corresponding to secretory-type OBM cDNA was collected by agarose gel electrophoresis. The fragment was inserted into an NheI/XhoI fragment (10.4 kb) of an expression vector pCEP4 (Invitrogen Co., Ltd.) by use of a ligation kit, and *Escherichia coli* DH5α were transformed by use of the reaction product. Plasmids were purified from the obtained ampicillin-resistant clones by an alkaline SDS method and cleaved by restriction enzymes and analyzed so as to select a *Escherichia coli* clone having a secretory OBM expression plasmid (pCEP sOBM) with the target structure. The *Echerichia coli* clone having the pCEP sOBM was cultured, and the pCEP sOBM was purified by use of a QIA® Filter Plasmid Midi Kit (QIAGEN Co., Ltd.). Next, 293-EBNA cell was suspended in IMDM containing 10% FCS (IMDM-10%FCS) and seeded in a collagen-coated 24 well plate (Sumitomo Bakelite Co., Ltd.) in an amount of 2 X 10^5 cells/2 ml/well, and cultured overnight. To the cells, 1 μg of pCEP sOBM or pCEP4 was transfected by use of 4 μl of lipofectamine (Gibco Co., Ltd.), and the cells were cultured for another 2 days in 0.5 ml of serum-free IMDM or IMDM-10%FCS, thereby collecting a conditioned medium. The clones with high production of recombinant mouse soluble OBM (msOBM) or human soluble OBM (hsOBM) were screened in the following manner. After sodium hydrogen carbonate was added to the conditioned medium seemed to contain msOBM or hsOBM at a final concentration of 0.1 M, 100 μl of the conditioned medium was added to each well of 96 well immunoplate (Nunc Co., Ltd.) and the plate was left to stand at 4°C overnight so as to solid-phase the msOBM or hsOBM in the conditioned medium on each well. Then, 200 μl of BLOCKACE (Snow Brand Milk Products Co., Ltd.) solution diluted to 4 times with PBS (B-PBS) was added to each well of the plate and the plate was left to stand at room temperature for 2 hours. After washing three times with PBS containing 0.1% Polysorbate 20 (P-PBS), 100 μl of recombinant OCIF (rOCIF) solution diluted stepwise (0-100 ng/ml) with B-PBS was added to each well and the plate was left to stand at 37°C for 2 hours. After washing three times with PBS, 100 μl of peroxidase labeled anti-OCIF polyclonal antibody (WO 96/26217), diluted with B-PBS, was added to each well and the plate was left to stand at 37°C for 2 hours. After washing six times with P-PBS, 100 μl of TMB solution (TMB Soluble Agent, High sensitivity, Scytek Co., Ltd.) was added to each well and left to stand at room temperature for about 10 minutes. Thereafter, 100 μl of Stopping Reagent (Scytek Co., Ltd.) was added to each well. The absorbance of each well at 450 nm was measured by

means of a microplate reader. In the plate having the solid-phased protein derived from conditioned medium of the clone producing msOBM or hsOBM, the absorbance significantly increased in proportion to the concentration of the OCIF. As for the clones producing msOBM or hsOBM, clones indicating a high rate of increase in the absorbance were selected as highly producing clones thereof. Each of the highly producing clones of msOBM or hsOBM selected in the above mentioned manner were mass-cultured by use of IMDM containing 5% FCS as a medium in 25 T-flasks (T-225). After the cells grew to confluency, 100 ml of fresh medium was added to each T-255 flask and the cells were further cultured for 3 or 4 days, and then a conditioned medium was collected. By repeating this procedure 4 times, 10 liters of the conditioned medium containing msOBM and 10 liters of the conditioned medium containing hsOBM were obtained. About 10 mg of purified msOBM and about 12 mg of purified hsOBM, which were uniform (molecular weight: 32 kDa) in terms of SDS-polyacrylamide electrophoresis, were obtained by carrying out purification on the above-obtained conditioned medium with affinity chromatography using an rOCIF-immobilized column and gel filtration chromatography in accordance with the method described in Examples 25-(6) and (7). The obtained purified samples were used as immunizing antigens. The obtained antigens each were dissolved in phosphate buffered saline (PBS) at a concentration of 200 μg/ml and then the solution was mixed with an equal amount of Freund's complete adjuvant so as to be emulsified. 1 ml of each emulsion was subcutaneously administered to three Japanese white rabbits at intervals of about one week so as to immunize the rabbits. An antibody titer was measured, and when the antibody titer reached a maximum, a booster was carried out. 10 days after the booster, all blood was collected from all the rabbits. Antiserum was diluted to two times with binding buffer for Protein A Sepharose® chromatography (Bio-Rad Co., Ltd.) and then added to a Protein A column equilibrated with the above buffer. After the column was efficiently washed with the above buffer, an anti-sOBM antibody adsorbed to the column was eluted by an elution buffer (Bio-Rad Co., Ltd.) or 0.1 M glycine-HCl buffer (pH 2.9 to 3.0). In order to immediately neutralize the antibody-containing eluate, the eluted solution was fractionated by use of a test tube containing a small amount of 1.0 M Tris-HCl (pH 8.0). The antibody eluate was dialyzed in PBS at 4°C overnight. The amount of protein in the antibody solution was measured in accordance with the Lowry method using bovine IgG as a standard. Thus, the purified immunoglobulin (IgG) containing the polyclonal antibody of the present invention was obtained in an amount of about 10 mg per 1 ml of rabbit antiserum.

[Example 29]

Measurements of OBM and sOBM by ELISA Using Polyclonal Antibody

Sandwich ELISAs, using the rabbit anti-hsOBM polyclonal antibody obtained

in Example 28 as a solid phase antibody and as an enzyme labeled antibody, were constructed. As enzyme labeling, peroxidase (POD) labeling was carried out in accordance with a method of Ishikawa *et al.* (Ishikawa *et al.*: J. Immunoassay, Vol. 4, 209 to 327, 1983). The anti-hsOBM polyclonal antibody obtained in Example 28 was dissolved in a 0.1 M $NaHCO_3$ solution at a concentration of 2 μg/ml, and 100 μl of the resulting solution was added to each well of 96-well immunoplate (Nunc Co., Ltd.) and the plate was left to stand at room temperature overnight. Then, 200 μl of 50% BLOCKACE (Snow Brand Milk Products Co., Ltd.) was added to each well, and the plate was left to stand at room temperature for 1 hour. Each well was washed with PBS containing 0.1% polysorbate 20 (washing buffer) three times. The purified human OBM, which was expressed in the same manner as in Example 26 and was purified in the same manner as in Example 2, and the purified human sOBM, obtained in Example 28, was diluted stepwise with primary reaction buffer (0.2 M Tris-HCl buffer containing 40% BLOCKACE and 0.1% polysorbate 20, pH 7.2), and 100 μl of each diluent were added to each well. After the plate was left to stand at room temperature for 2 hours, each well was washed with the above washing buffer three times. 100 μl of POD labeled anti-human sOBM polyclonal antibody, diluted 1,000 times with secondary reaction buffer (0.1 M Tris-HCl buffer containing 25% BLOCKACE and 0.1% polysorbate 20, pH 7.2) was added to each well and the plate was left to stand at room temperature for 2 hours. Each well was washed with the washing buffer three times. 100 μl of substrate solution (TMB, ScyTek Co., Ltd.) was added to each well, and the plate was left to stand at room temperature for 10 minutes. 100 μl of reaction stopping solution (Stopping reagent, ScyTek Co., Ltd.) was added to each well so as to stop the enzyme reaction. The absorbance at 450 nm of each well was measured by use of a microplate reader. The results are shown in Fig. 26. The sandwich ELISA, using the rabbit anti-human sOBM polyclonal antibody, almost equally detected both human sOBM (molecular weight: about 32 kDa) and human OBM (molecular weight: about 40 kDa), and measurement sensitivity was about 12.5×10^{-3} pmol/ml (about 500 pg/ml for human OBM, about 400 pg/ml for human sOBM). It was revealed that measurements of mouse sOBM and mouse OBM by ELISA using the rabbit anti-mouse sOBM polyclonal antibody obtained in Example 28 could be made in the same manner as described above, measurement sensitivity in measuring mouse OBM or mouse sOBM was similar with that in human OBM or human sOBM, and a very small amount of mouse sOBM or mouse OBM could be measured.

As described above, since the present anti-human sOBM polyclonal antibody obtained in Example 28 recognized both human sOBM and human OBM as antigen equally, it was named an anti-human OBM/sOBM polyclonal antibody. Meanwhile, since the anti-

mouse sOBM polyclonal antibody obtained in Example 28 recognized both mouse sOBM and mouse OBM as antigen equally, it was named an anti-mouse OBM/sOBM polyclonal antibody.

[Example 30]

Preparation of Monoclonal Antibody

The purified human sOBM obtained in Example 28 was used as an immunizing antigen. The purified human sOBM was dissolved in phosphate buffered saline at a concentration of 10 μg/ml. To the prepared human sOBM solution, an equal amount of Freund's complete adjuvant was added so as to emulsify it. Thereafter, 200 μl of the antigen was administered into the abdominal cavity of each Balb/c mouse at an interval of one week for a total of three times so as to immunize the mice. Then, to a physiological saline solution containing 5 μg/ml of the human sOBM, an equal amount of Freund's incomplete adjuvant was added so as to fully emulsify it, and 200 μl of the emulsion was administered to each of the above Balb/c mice at one week intervals for a total of four times so as to further immunize the mice. After the passage of one week from the fourth additional immunization, 100 μl of phosphate buffered saline solution containing 10 μg/ml of the human sOBM was parenterally administered to each of the Balb/c mice for booster. On the 3rd day after the final immunization, the spleen was removed, and spleen cells were separated and fused with mouse myeloma cells P3x63-AG8.653 in accordance with a known method (Koehler, G. and Milstein, C., Nature, 256, 495 (1975)). After completion of the fusion, the cell suspension was cultured in a HAT medium containing hypoxanthine, aminopterin and thymidine for 10 days. After the myeloma cells perished and hybridomas appeared, the medium was replaced with an HT medium obtained by removing aminopterin from the HAT medium, and the culture was continued.

[Example 31]

Selection and Cloning of Hybridoma

Since the appearance of the hybridoma was seen on the 10th day from the start of the cell fusion and culturing in Example 30, a high affinity antibody recognizing human sOBM and hybridoma producing the antibody were selected in the means of the following improved solid phase ELISA. Further, to select an anti-OBM monoclonal antibody recognizing both of human sOBM and mouse sOBM, the mouse sOBM obtained in Example 27 as well as human sOBM was used as an antigen in the solid phase ELISA. Human sOBM and mouse sOBM each was dissolved in 0.1 M sodium hydrogen carbonate solution at a concentration of 5 μg/ml, and 50 μl of each antigen solution was added to each well of a 96-well immunoplate (Nunc Co., Ltd.), and the plate was left to stand at 4°C overnight so as

to attach the antigens. The antigen solution in each well was discarded, and 200 μl of 50% BLOCKACE (Snow Brand Milk Products Co., Ltd.) was added to each well. The plate was left to stand at room temperature for 1 hour so as to cause blocking. After each well was washed with a phosphate buffered saline containing 0.1% polysorbate 20, 40 μl of bovine serum (Hyclone Co., Ltd.) was added to each well. Then, 10 μl of hybridoma conditioned medium was added to each well and the plate was left to stand under a serum concentration of 80% at room temperature for 2 hours so as to cause reaction. An object of the solid phase ELISA in the presence of 80% serum is to select an antibody capable of binding to a small amount of human sOBM or mouse sOBM even in the presence of protein and a serum-derived immune reaction inhibiting substance in high concentration, that is, to select a hybridoma producing an antibody having high affinity for human sOBM or mouse sOBM. After completion of the reaction at room temperature for 2 hours, the plate was washed with PBS-P, and 50 μl of diluent of peroxidase labeled anti-mouse IgG (KPL CO., LTD.) diluted to 5,000 times with a physiological saline solution containing 25% BLOCKACE was added to each well, and the plate was left to stand at room temperature for 2 hours so as to cause a reaction. After the plate was washed with PBS-P three times, 50 μl of substrate solution (TMB, ScyTek Co., Ltd.) was added to each well and left to stand at room temperature for 5 minutes. Then, 50 μl of a reaction stopping reagent (Stopping Reagent, ScyTek Co., Ltd.) was added so as to terminate the enzyme reaction. The absorbance at 450 nm of each well was measured by use of a microplate reader (IMMUNOREADER NJ2000, Nippon Intermed Co., Ltd.) so as to select a hybridoma producing an antibody which recognizes human sOBM or mouse sOBM. The hybridomas showing particularly high absorbance (OD_{450nm}) were selected and repeatedly cloned 3 to 5 times by a limiting dilution method so as to establised hybridomas producing antibody stably. Out of the obtained hybridomas, hybridomas having higher antibody productivity were selected.

[Example 32]

Production and Purification of Monoclonal Antibody

The antibodies obtained in Example 31, that is, the hybridoma producing an antibody which recognizes human sOBM with high affinity and the hybridoma producing the antibody which has a cross-reactivity with mouse sOBM were cultured, and each hybridoma was implanted in the abdominal cavity of a Balb/c-based mouse which had been given pristane (Aldrich Chemical Co., Ltd.) about a week before, in an amount of 1×10^6 cells/mouse. After about 2 or 3 weeks, accumulated ascites was sampled so as to obtain ascites containing the monoclonal antibody recognized human sOBM or the monoclonal antibody recognizing human sOBM and mouse sOBM. Purified monoclonal antibodies were

obtained from the ascites using Protein A column (Pharmacia Co., Ltd.) chromatography in accordance with the method for purifying an anti-OBM/sOBM polyclonal antibody described in Example 28.

[Example 33]

Antigenic Specificity of the Monoclonal Antibody

The antigenic specificities of monoclonal antibodies, which specifically recognized human sOBM, and of monoclonal antibodies, having cross-reactivity with human sOBM and mouse sOBM, were examined using human sOBM, intact human OBM having a membrane binding site, mouse sOBM, and intact mouse OBM having a membrane binding site as antigens. Although over 30 types of monoclonal antibodies were obtained, the results of representative monoclonal antibodies are shown in Table 1. As a result, it was revealed that most of anti-human sOBM monoclonal antibodies which specifically recognized human sOBM recognized even intact human OBM having a membrane binding site and did not recognize mouse sOBM and intact mouse OBM having a membrane binding site.

Meanwhile, a few monoclonal antibodies recognizing both of human sOBM and mouse sOBM were also obtained and it was found that these antibodies had cross-reactivity with human OBM and mouse OBM. These results indicate that human OBM and mouse OBM had a common antigen recognition site, i.e., epitope. Since an anti-human sOBM monoclonal antibody prepared by use of human sOBM as an antigen also equally recognized human OBM, which was a membrane binding intact protein, the monoclonal antibody was named an anti-human OBM/sOBM monoclonal antibody.

Table 1

	Antigen			
Antibody	hsOBM	hOBM	msOBM	mOBM
H-OBM 1	+	+	-	-
H-OBM 2	+	+	-	-
H-OBM 3	+	+	-	-
H-OBM 4	+	+	-	-
H-OBM 5	+	+	-	-
H-OBM 6	+	+	-	-
H-OBM 7	+	+	-	-
H-OBM 8	+	+	-	-
H-OBM 9	+	+	+	+
H-OBM 10	+	+	-	-
H-OBM 11	+	+	-	-
H-OBM 12	+	+	-	-
H-OBM 13	+	+	+	+
H-OBM 14	+	+	-	-

(hsOBM: human soluble OBM, hOBM: human membrane binding OBM, msOBM: mouse soluble OBM, mOBM: mouse membrane binding OBM)

[Example 34]

Tests of Class and Subclass of Monoclonal Antibody

The class and subclass of the monoclonal antibody of the present invention were determined by use of the Immunoglobulin Class/Subclass Analytical Kit (Amersham Co., Ltd.). The tests were conducted in accordance with a protocol provided in the kit. The results of representative monoclonal antibodies are shown in Table 2. The majority of anti-human OBM/sOBM monoclonal antibodies had IgG_1, and some antibodies having IgG_{2a} or IgG_{2b} were also found. Further, all of the antibodies had κ chain as a light chain.

Table 2

Antibody	IgG_1	IgG_{2a}	IgG_{2b}	IgG_3	IgA	κ
H-OBM 8	-	+	-	-	-	+
H-OBM 9	+	-	-	-	-	+
H-OBM 10	+	-	-	-	-	+
H-OBM 11	+	-	-	-	-	+
H-OBM 12	-	-	+	-	-	+
H-OBM 13	+	-	-	-	-	+
H-OBM 14	+	-	-	-	-	+

[Example 35]

Measurement of Dissociation Constant (K_D value) for the Monoclonal Antibodies

The dissociation constants for monoclonal antibodies were measured in accordance with a known method (Betrand Friguet *et al.*: Journal of Immunological Methods, 77, 305 to 319, 1986). That is, the purified antibody obtained in Example 32 was diluted at 5 ng/ml with 0.4 M Tris-HCl containing 40% BLOCKACE and 0.1% polysorbate 20 (pH 7.4, primary buffer) and an equal amount of diluent of the purified human soluble OBM (hsOBM) obtained in Example 28, prepared with the primary buffer at stepwise-concentration from 6.25 ng/ml to 10 μg/ml, was added and the solution was left to stand at 4°C for 15 hours so as to bind the monoclonal antibody to hsOBM. After 15 hours, an antibody unbound to hsOBM was measured by solid phase ELISA with solid-phased hsOBM (10 μg/ml, 100 μl/well) so as to calculate the dissociation constant of the monoclonal antibody to hsOBM. Further, the affinity for msOBM of monoclonal antibodies, having cross-reactivity with mouse soluble OBM (msOBM) and hsOBM, was also measured by using msOBM in place of hsOBM at the above-mentioned method. Particularly, the results of particular antibodies, which had high affinity for each of the antigens and were useful in enzymatic immunoassay, binding assay and such, are shown in Table 3.

Table 3

Antibody	Subclass	Antigen	Dissociation Constant Kd (M)
H-OBM 1	IgG_1 (κ)	hsOBM	$1 \times 10^{-11} < Kd < 1 \times 10^{-10}$
H-OBM 4	IgG_1 (κ)	hsOBM	$1 \times 10^{-11} < Kd < 1 \times 10^{-10}$
H-OBM 9	IgG_1 (κ)	hsOBM	$1 \times 10^{-9} < Kd < 1 \times 10^{-8}$

H-OBM 9	IgG_1 (κ)	msOBM	$1 \times 10^{-8} < Kd < 1 \times 10^{-7}$

As a result, it was found that H-OBM 1 and H-OBM 4 which were specific antibodies for human soluble OBM (hsOBM) showed a dissociation constant on the order of 10^{-11} M, indicating that they had very high affinity for hsOBM. Meanwhile, the Kd value of H-OBM 9 which was an antibody recognizing both hsOBM and mouse soluble OBM (msOBM) was on the order of 10^{-8} M with respect to msOBM and on the order of 10^{-9} M with respect to hsOBM. Further, regarding H-OBM 13, which was another antibody recognizing both antigens shown in Table 1, the dissociation constants of H-OBM 13 with respect to both antigens were almost identical with those of H-OBM 9, and since both antibodies had the same subclass, a possibility was suggested that they were the same antibody recognizing the same epitope.

[Example 36]

Method for Measuring Human OBM and sOBM by Sandwich ELISA Using Anti-Human OBM/sOBM Monoclonal Antibody

Sandwich ELISA was constructed by use of the two types of high affinity monoclonal antibodies obtained in Example 35, i.e., H-OBM 1 and H-OBM 4, as a solid phase antibody and an enzyme labeled antibody, respectively. Maleimide Activated Peroxidase Kit (Pierce Co., Ltd.) was used for labeling the antibody. H-OBM 1 antibody was dissolved in 0.1 M sodium hydrogen carbonate solution at a concentration of 10 μg/ml, and 100 μl of the resulting solution was added to each well of 96-well immunoplate (Nunc Co., Ltd.). The plate was left to stand at 4°C overnight so as to attach the antibody. After the solution in each well was discarded, 300 μl of 50% BLOCKACE was added to each well, and the plate was left to stand at room temperature for 2 hours so as to cause blocking. After the blocking, the plate was washed with phosphate buffered saline containing 0.1% polysorbate 20 (PBS-P). Human soluble sOBM and human OBM each were dissolved in 0.4 M Tris-HCl (pH 7.4) containing 40% BLOCKACE (Snow Brand Milk Products Co., Ltd.) and 0.1% polysorbate 20 (Wako Pure Chemical Industries, Ltd.) (primary reaction buffer) and diluted so as to prepare test samples with various concentrations. 100 μl of each of test sample, prepared at various concentrations, was added to each well, and the plate was left to stand at room temperature for 2 hours so as to cause a reaction. Thereafter, the plate was washed with PBS-P, and 100 μl of POD labeled H-OBM 4 antibody diluted with 0.2 M Tris-HC (pH 7.4) containing 25% BLOCKACE and 0.1% polysorbate 20 (secondary reaction buffer) was added to each well. The plate was left to stand at room temperature for 2 hours so as to cause a reaction. After the plate was washed with PBS-P, 100 μl of substrate solution (TMB,

ScyTek Co., Ltd.) was added to each well so as to develop color in the wells, and 100 μl of reaction stopping solution (stopping reagent, ScyTek Co., Ltd.) was added to each well so as to stop the enzyme reaction. The absorbance at 450 nm of each well was measured by use of a microplate reader. The results are shown in Fig. 27.

As a result, it was revealed that the sandwich ELISA constructed by use of the two types of high affinity anti-human OBM/sOBM monoclonal antibodies obtained in Example 35, i.e., H-OBM 1 and H-OBM 4, detected human OBM and human sOBM equally. The measurement sensitivity thereof was about 1.25 to 2.5 X 10^{-3} pmol/ml (about 50 to 100 pg/ml for human OBM having a molecular weight of about 40 kDa, about 40 to 80 pg/ml for human sOBM having a molecular weight of about 32 kDa), and very small amounts of human OBM and human sOBM could be measured by the ELIZA. Hybridomas producing these two types of anti-human OBM/sOBM monoclonal antibodies, H-OBM 1 and H-OBM 4, were named H-OBM1 and H-OBM4, respectively. Further, a hybridoma producing H-OBM 9, the anti-human OBM/sOBM monoclonal antibody which recognized both mouse OBM and mouse sOBM and exhibited osteoclastogenesis inhibitory activity, was named H-OBM9. These hybridomas were deposited with the National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry with deposit numbers FERM BP-6264 (H-OBM1), FERM BP-6265 (H-OBM4) and FERM BP-6266 (H-OBM9) on November 5, 1997.

[Example 37]

Measurements of Mouse OBM and Mouse sOBM Using Anti-Human OBM/sOBM Monoclonal Antibody Recognizing Mouse OBM and Mouse sOBM

Sandwich ELISAs using the anti-human OBM/sOBM monoclonal antibody H-OBM9 recognizing mouse OBM and mouse sOBM and obtained in Examples 33 and 35 as a solid-phased antibody, and using the anti-mouse OBM/sOBM polyclonal antibody obtained in Example 28 as an enzyme labeled antibody, were constructed. Mouse OBM and mouse sOBM were diluted stepwise with the primary reaction buffer in the same manner as in Example 35, and the mouse OBM and mouse sOBM were detected in the same manner as in Example 36. The results are shown in Fig. 28. As a result, it was confirmed that the mouse OBM and mouse sOBM could be detected equally by use of the anti-human OBM/sOBM monoclonal antibody H-OBM 9 which recognized the mouse OBM and mouse sOBM. As shown in the results of Example 35, the antibody H-OBM 9 had a high dissociation constant with respect to the mouse sOBM; in other words, the antibody had relatively low affinity for the mouse sOBM. Thus, the measurement sensitivities of mouse OBM (molecular weight: about 40 kDa) and mouse sOBM (molecular weight: about 32 kDa) by the above ELISA were about 25 X 10^{-3} pmol/ml (about 1 ng/ml for mouse OBM, about 0.8 ng/ml for mouse sOBM).

[Example 38]

Assay for Osteoclastogenesis Inhibitory Activity of Anti-OBM/sOBM Antibody

It is known that an osteoclast-like cell (OCL) is derived by co-culture of mouse spleen cell and ST2 cell (mouse bone marrow derived interstitial cell) (Endocrinology, 125, 1,805 to 1,813 (1989)). Thus, it was examined whether derivation of OCL was inhibited by addition of an OBM/sOBM antibody to the co-culture. Since mouse OBM was expressed in the co-culture system, antibodies used in this Example were H-OBM 9 and rabbit anti-mouse OBM/sOBM polyclonal antibody recognizing mouse OBM. The OBM antibodies each were diluted stepwise with αMEM containing 10% FCS and added to a 24 well plate (Nunc Co., Ltd.) in an amount of 700 μl/well, and male mouse spleen cells suspended in the above medium (2×10^6/ml) were also added in an amount of 350 μl/well. Then, trypsinized ST2 cells were suspended (8×10^4 cells/ml) in the above medium containing 4×10^{-8} M vitamin D_3 and 4×10^{-7} M dexamethasone, and the resulting suspension was added in an amount of 350 μl/well. The plate was incubated at 37°C for 6 days for culture. After the plate was washed with PBS once, the cells were fixed by mixture of 50% ethanol and 50% acetone at room temperature for a minute. After the plate was air-dried, substrate solution was added in an amount of 500 μl/well in accordance with a protocol of a leukocyte acid phosphatase kit (Sigma Co., Ltd.), and the plate was left to stand at 37°C for 55 minutes so as to cause reaction. By this reaction, cell showing tartaric acid resistant acid phosphatase activity (TRAP activity), which is a specific marker of osteoclasts, were stained. After the plate was washed with distilled water once and air-dried, the number of TRAP positive cells were counted. The results are shown in Table 4. As a result, it was found that both of the rabbit anti-mouse OBM/sOBM polyclonal antibody and H-OBM 9 inhibited derivation of OCL depending on the concentrations of the antibody. It was found that these antibodies had osteoclastogenesis inhibitory activity as in the case of an osteoclastogenesis factor, OCIF/OPG, and were useful as a medicament for treating bone metabolism abnormality.

Table 4

Amount of Antibody Added (ng/ml)	Number of TRAP Positive Multinucleate Cells	
	Rabbit Anti-Mouse OBM/sOBM Polyclonal Antibody	Mouse Anti-Human OBM/sOBM monoclonal antibody (H-OBM 9)
0	1,155 ± 53	1,050 ± 45
10	510 ± 24	650 ± 25
100	10 ± 3	15 ± 4

(average ± standard deviation, n = 3)

[Example 39]

Osteoclastoginesis Inducing Activity of Trx-OBM

Mononuclear cells were prepared from whole blood sampled from a vein of a normal adult human using Histopaque (Sigma Co., Ltd.) with density gradient technique in accordance with an attached protocol. The mononuclear cells were suspended at a concentration of 1.3 X 10^6cells/ml with α-MEM containing 10^{-7} M dexamethasone, 200 ng/ml of macrophage colony stimulating factor (Midori Juji Co., Ltd.), 10% fetal bovine serum and stepwise concentration (0 to 100 ng/ml) of purified Trx-OBM obtained in Example 15. The suspension was added to a 48-well plate in an amount of 300 μl/well, and the plate was incubated at 37°C for 3 days for culturing cells. Thereafter, the medium was replaced with new (identical with above), and the plate was incubated at 37°C for another 4 days for culturing cells. Cell showing tartaric acid resistant acid phosphatase activity (TRAP activity) were selectively stained by the method described in Example 5, and the number of stained multinuclear cells was counted under the microscope. The results are shown in Fig. 29. As a result, cells showing TRAP activity were hardly detected in the wells containing no Trx-OBM, while TRAP positive multinuclear cells appeared in a manner depend on concentration of Trx-OBM when Trx-OBM was added. Further, these TRAP positive multinuclear cells showed positive result for vitronectin receptor which is a marker of osteoclasts. In addition, when the same culture conditions were used on dentin fragments placed on a 48-well plate, absorption cavities were formed on the surface of dentin fragments only in the presence of Trx-OBM. Thereby, it was revealed that Trx-OBM had activity to induce formation of human osteoclasts.

[Example 40]

Bone Resorption Inhibitory Activity of Anti-OBM/sOBM Antibody

15-day pregnant ddy mice (Nippon SLC Co., Ltd.), 25 μCi of [^{45}Ca]-$CaCl_2$ solution (Amersham Co., Ltd.) were injected subcutaneously, and fetal bones were labeled with ^{45}Ca. On the following day, the mice were slaughtered, and their abdomens were opened to remove fetuses from the uteruses. A forelimb was removed from the fetus, the skin and muscle were removed to take out a long bone, and a cartilage on the long bone was also removed so as to leave only the diaphysis of the long bone. Each diaphysis was floated in 0.5 ml of culture medium (BGJb medium (Gibco Co., Ltd.) containing 0.2% bovine serum albumin (Sigma Co., Ltd.)) and cultured at 37°C in the presence of 5%CO_2 for 24 hours. After completion of the pre-culture, the long bone was transferred to a new culture medium (0.5 ml) containing various bone resorption factors (vitamin D_3, prostaglandin E_2, parathyroid hormone, interleukin 1α) and normal rabbit IgG (100 μg/ml; as a control) or the rabbit anti-OBM/sOBM polyclonal antibody obtained in Example 28, and then cultured for another 72

hours. After completion of the culture, the long bone was put into 0.5 ml of 5% trichloroacetic acid aqueous solution (Wako Pure Chemical Industries, Ltd.) and treated at room temperature for at least 3 hours so as to be decalcified. To the conditioned medium and the trichloroacetic acid extract (0.5 ml each), 5 ml of scintillator (AQUASOL-2, Packard Co., Ltd.) was added and the radioactivity of ^{45}Ca was measured. The proportion of ^{45}Ca liberated in the culture solution due to bone resorption was calculated. The results are shown in Figs. 30 to 33. As a result, although the vitamin D_3 (10^{-8} M) caused increase of bone resorption activity, the bone resorption caused by the vitamin D_3 was inhibited by addition of the rabbit anti-OBM/sOBM polyclonal antibody in a concentration-dependent manner, and the bone resorption was completely inhibited by addition of the antibody at a concentration of 100 μg/ml (Fig. 30). Further, although bone resorption activity was increased in the presence of prostaglandin E_2 (10^{-6} M) or the parathyroid hormone (100 ng/ml), the bone resorption caused by the prostaglandin E_2 or the parathyroid hormone was almost completely inhibited by the addition of the rabbit anti-OBM/sOBM polyclonal antibody (100 μg/ml)(Figs. 31 and 32). Meanwhile, the normal rabbit IgG (100 μg/ml) used as a positive control had no effects on the bone resorption by the prostaglandin E_2 and the parathyroid hormone. Further, although bone resorption was induced by the interleukin 1α (10 ng/ml) as well, the bone resorption was inhibited significantly by the rabbit anti-OBM/sOBM polyclonal antibody (100 μg/ml) (Fig. 23). From these results, it was revealed that the antibody of the present invention was excellent as a bone resorption inhibitory substance. As a result of conducting the same experiment on H-OBM 9 which was a mouse anti-human OBM/sOBM antibody, it was confirmed that H-OBM 9 had approximately equal bone resorption inhibitory activity to that of the rabbit anti-OBM/sOBM polyclonal antibody.

Industrial Applicability

The present invention provides a novel protein which binds osteoclastogenesis inhibitory factor (OCIF), a method for production thereof, a method for screening a substance which controls expression of the protein by use of the protein, a method for screening a substance which inhibits or modifies an activity of the protein, a method for screening a receptor which binds the protein and transmits an activity thereof, a pharmaceutical composition comprising a substance obtained by said method for screening, an antibody to the protein, and an agent for treating bone metabolism abnormality which is formulated using the antibody.

Furthermore, the present invention provides a DNA which encodes a novel protein (OCIF binding molecule) which binds osteoclastogenesis inhibitory factor (OCIF), a

protein having an amino acid sequence encoded by the DNA, a method for genetically producing a protein which specifically binds to the OCIF by use of the DNA, and an agent for treating bone metabolism comprising the protein. Moreover, methods are provided for screening a substance which controls the expression of the OCIF binding molecule, a method for screening a substance which binds to the OCIF binding molecule and inhibits or modifies an activity thereof, a method for screening a receptor which binds OCIF binding molecule and transmits an activity thereof, and a pharmaceutical composition comprising a substance obtained by said method for screening.

Also provided is: DNA, which encodes a novel human protein (human-derived OCIF binding molecule, human OBM) which binds osteoclastogenesis inhibitory factor (OCIF), a protein and having an amino acid sequence encoded by the DNA, a method for genetically producing a protein which specifically binds OCIF and has a biological activity to support and promote the differentiation and maturation of osteoclasts by use of the DNA, and an agent for treating bone metabolism abnormality comprising the protein.

Also provided are: a method for screening a substance which controls expression of the OCIF binding molecule, a method for screening a substance which binds the OCIF binding molecule and inhibits or modifies an activity thereof, a method for screening a receptor which binds the OCIF binding molecule and transmits the biological activity thereof, and a pharmaceutical composition comprising a substance obtained by said method for screening, as well as an antibody to the human-derived OCIF binding protein, and an agent for preventing and/or treating bone metabolism abnormality which is formulated using the antibody.

Moreover, the present invention provides an antibody (anti-OBM/sOBM antibody) which recognizes both of the following antigens, i.e., a membrane binding molecule (OCIF binding molecule; OBM) which specifically binds to an OCIF, and a soluble OBM (sOBM) lacking membrane binding sites, a method for production of the antibody, a method for measuring the OBM and sOBM by use of the antibody, and an agent for preventing and/or treating bone metabolism abnormality which comprise the antibody as an active ingredient.

The proteins or antibodies presented by the present invention are useful as medicaments, experimental reagents or diagnostic reagents.

Reference to Deposited Microorganisms

(1) Name and Address of Depository Institution:

National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry

1-1-3 Higashi, Tsukuba-shi, Ibaragi-ken, Japan (zip: 305)

Date of Deposit:

May 23, 1997

Deposit Number:

FERM BP-5953

(2) Name and Address of Depository Institution:

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August 13, 1997

Deposit Number:

FERM BP-6058

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Date of Deposit:

November 5, 1997

Deposit Number:

FERM BP-6264

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November 5, 1997

Deposit Number:

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Date of Deposit:

November 5, 1997

Deposit Number:

FERM BP-6266